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HORMONES AND THE TERMINATION AND REINDUCTION OF DIAPAUSE IN *CEPHUS CINCTUS* NORT. (HYMENOPTERA: CEPHIDAE)[1]

BY NORMAN STANLEY CHURCH[2]

Abstract

Ligation and parabiosis experiments and cytological studies showed post-diapause morphogenesis in *Cephus cinctus* larvae to be initiated by a growth and differentiation hormone from the prothoracic glands. The prothoracic glands in turn are probably stimulated by a hormone from the protocerebral neurosecretory cells. The brain continues to influence the prothoracic glands even after they have begun to secrete and the influence is more than that of a simple stimulation by the neurohormone. Furthermore, experiments with larvae reared apart from their cocoons suggested that the brain is to some extent influenced by environmental stimuli. If an effective amount of growth and differentiation hormone has not yet been secreted, exposure of a week or less at 35° C. causes postdiapause *C. cinctus* larvae to revert to diapause. Forty degrees has a much weaker effect. Cytological examination suggested that 35° C. halts prothoracic gland activity and, meanwhile, permits the neurohormone to be dissipated. At the end of the heat treatment there is insufficient neurohormone left to reactivate the prothoracic glands, which revert to dormancy.

Introduction

Wigglesworth (47), over 20 years ago, proposed that diapause may be primarily the result of a hormone failure. This viewpoint is gaining increasing support.

The Hormonal Control of Molting

It has been proved that molting, including that by which a pupa or adult is formed, depends for its initiation on a "growth and differentiation" hormone secreted by a pair (usually) of diffuse organs, the prothoracic glands. These have variously been called thoracic glands, ventral glands, corpora incerta, subesophageal glands, and hypostigmatic glands (43). They are generally situated in the prothorax but may extend into the head or mesothorax. These glands or their homologues have been seen in Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Blattaria, Phasmida, Mantodae, Odonata,

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Ephemerida, Plecoptera, Dermaptera, and Isoptera. They exist in Diptera as the giant lateral cells, commonly called peritracheal or pericardial cells, of the ring gland. Work on this subject has been reviewed by Scharrer (35) and Williams (54, 56). By ligation, gland extirpation and implantation, and blood transfusion experiments the control over molting exerted by the prothoracic glands has been demonstrated in *Bombyx* (11) and *Platysamia* (53) among the Lepidoptera, in Diptera, Odonata, and Phasmida, and finally in the hemipteron *Rhodnius* (51). Wigglesworth (50) has reviewed most of this work.

Kopec (24) discovered in *Lymantria* that another organ, the brain, controlled the initiation of molting, apparently by way of glandular action. Since then, dependence of molting upon the brain has been demonstrated in other Lepidoptera, in Hemiptera, Orthoptera, Phasmida, Coleoptera, and Diptera (reviewed by Wigglesworth (49, 50)), and in Hymenoptera (39). In some species the situation has been more precisely defined: the brain is the source of a stimulus that activates the prothoracic glands. A small number of neurosecretory cells in the pars intercerebralis of the protocerebrum secrete a hormone that, when it reaches the prothoracic glands, causes them to begin their secretion. This series of events has been traced in *Rhodnius* and *Platysamia* by the classic experiments of Wigglesworth (47, 48, 50, 51) and of Williams (52, 53, 57), in *Calliphora* by Possompes and in *Bombyx* by Bounhiol (5, 6) and Fukuda (11). The sole function of the neurosecretory cells at this stage appears to be the stimulation of the prothoracic glands. Once this has been done the secretory activity of the prothoracic glands and the molting process are no longer dependent upon the brain.

Protocerebral neurosecretory cells have been revealed histologically in Hymenoptera, Lepidoptera, Coleoptera, Diptera, Trichoptera, Orthoptera, Hemiptera, and Neuroptera (reviewed by Day (9) and Scharrer and Scharrer (38)), and in Plecoptera (2). Their homologues have been found in the Apterygota (18). In several cases their activity has been correlated with the occurrence of molting.

Similar neurosecretory cells have been found associated with endocrine systems in vertebrates. Cytological examination indicates that neurosecretory cells, although they have acquired a capacity for hormone secretion, have not lost their original nervous function. Consequently Scharrer (37) believes that they serve as connecting links between the nervous and endocrine systems, thereby co-ordinating the control exerted by the two systems over the organism's physiology.

The Hormone Failure Theory of Diapause

Platysamia experiences a pupal diapause before the endocrine mechanism controlling its imaginal molt and metamorphosis is set in motion. Williams (52) demonstrated that during this diapause the implantation of brains from chilled postdiapause pupae was enough to activate the prothoracic glands, which by secreting the growth and differentiation hormone elicited a normal metamorphosis. Active brain implants also were effective in breaking diapause

in *Leptinotarsa* (17) and *Gryllus* (42). In these species diapause seems to result from failure of the hormone mechanism to operate, as Wigglesworth suggested. Furthermore, the brain's neurosecretory cells and not the prothoracic glands must be nearest the origin of the failure.

Schneider (41) has interpreted the function of the intercerebralis neurosecretory cells quite differently. In syrphid larvae during diapause they are periodically filled with secretory droplets, which disappear if the insects are moistened or warmed. A temporary activation immediately follows such treatment, and in *Syrphus ribesii* (L.) it is often enough to induce postdiapause development. Generally, especially in other syrphid species, the activation is soon superseded by an immobility even more rigid than before. From this Schneider concluded that the neurosecretory cells produce an inhibitory agent that causes diapause and sustains it. Nonetheless, one might just as reasonably hold the opposite view, that the release of stored secretion from the intercerebralis cells follows the stimulus of wetting or warming and causes the fleeting reactivation of the insect, but that for some reason the secretion is usually insufficient or the insect not yet receptive enough for it to bring an end to diapause.

L'Hélias (19) reported a different situation in *Lophyrus*. There the brain's secretory activity is restricted to actively developing stages and is absent during diapause.

In Andrewartha's opinion, since diapause (at least larval, nymphal, or pupal diapause) occurs near the end of a stadium, the hormone failure theory is probably generally applicable (1).

Significance of Other Theories of Diapause

If one accepts—cautiously—the hormone failure theory of diapause, the explanation is still far from complete. There has not yet been proposed a convincing reason for the failure of the hormones. Andrewartha believes that the brain does not release the hormone mechanism because the former is inhibited by the accumulation of reserve food in a form that is not immediately available to the tissues, and that diapause development is a process of food mobilization or processes prerequisite to it. The abundance of reports of marked differences in fat body and other tissues between diapause and nondiapause insects gives at least circumstantial support to this theory (1).

There are theories of diapause antedating the hormone failure concept that should not be completely forgotten. They have been reviewed fully and skillfully by Andrewartha (1), Lees (25, 26), Prebble (30), and Simmonds (44) in relation to a multitude of pertinent observations. The idea of substances in the body inhibiting development received considerable attention, particularly by Roubaud (31, 32) and Bodine (4), long before Andrewartha's food mobilization hypothesis. The inhibition was generally thought of as being at least a mildly pathological condition and more recent work has denied the theories any general applicability.

Yet one must not too eagerly dispose of the possibility that inhibitory substances do contribute to diapause in some way. They may be the agents whereby environmental conditions can influence diapause, particularly its inception. On second thought, however, it is probably unwise to consider such substances as merely inhibitory; they would be more likely to promote particular biological processes that lead to the diapause state, and at the same time hinder others. An important series of papers by Fukuda (12–15) gives evidence that such agents do exist, that there is a diapause-inducing factor secreted by the subesophageal ganglion in *Bombyx*. Its secretion in the female pupa determines that upon maturity the insect will lay diapause eggs. The brain suppresses or stimulates the production of this factor according to the genetic type and previous history of the insect.

Influence of Environment on Diapause

The environmental factors that can cause insects to enter diapause are many. Generally unfavorable conditions that slow the rate of development usually strengthen the tendency towards diapause in species that have a facultative diapause, i.e., in species in which each individual is not obliged to go into dormancy. Andrewartha (1) has critically reviewed the extensive literature on this subject. Deficient or excessive moisture, food quality and its dryness, low or unusually high temperatures, and overcrowding and isolation have all been implicated. Unfortunately, in some of the literature in which the influence of these factors is described, no distinction is made between (*a*) development that is arrested directly and only temporarily by some limiting condition and (*b*) real diapause, in which the arrest is less direct and development cannot be re-induced by simply removing the inhibitory agent and providing the kind of environment normally suitable for development.

There are other diapause-producing factors that ordinarily are not associated with an unfavorable environment. Among them are declining, but not necessarily low, temperatures (8), and relative lengths of dark and light periods (27). The fact that some of the factors that cause diapause are not in themselves unfavorable to development, and also that many diapause-inducing factors intensify as the mild growing season is about to give way to a less beneficent interval, both speak for an adaptive value for diapause. The idea that diapause insects are more able to survive cold or drought is fairly old but it is probably worth repeating. Certainly it is true of many species that diapause is more than just an unhealthy state caused by fatigue or an unfavorable environment from which the insect requires considerable time to recover. Whether or not this is considered may strongly influence the interpretation of experimental work.

One fact is very conspicuous and no doubt equally significant: the fact that the causative influence of environment on diapause is exerted by way of long and devious physiological processes. This is well demonstrated by the time

lapse between cause and observed effect (1). It is sometimes the next generation that is affected. The work of Kogure (23) on *Bombyx* is particularly striking. He found that light and temperature acting on incubating eggs partly determine whether or not eggs of the next generation will become dormant.

Diapause Development: The Elimination of Dormancy

On the basis of the hormone failure theory being generally applicable, two big problems remain. (*a*) By what agents do ecological factors become connected with the inhibition (whether it be active or passive) of the brain-prothoracic gland mechanism ? (*b*) What happens during the period of this inhibition, i.e., what is diapause development and how does it lead to the reactivation of the endocrine mechanism and the elimination of dormancy? What little is understood about the first question was discussed in the preceding sections. Here, some remarks on the second question may be of value even though we are as little able to answer it as we are the first.

It should be emphasized that, if the onset of diapause has been promoted by a certain environmental factor, the mere removal of or compensation for that factor does not break the dormancy. Low temperature is a common cause of diapause, yet nearly always the best temperature for diapause elimination is 10° to 25° C. lower than the morphogenetic optimum.

Diapause breaking is a progressive process. The rate–temperature curves for diapause elimination and for normal nondiapause development are similar, except that the entire curve for the former is displaced towards the lower end of the temperature scale. In both normal development and diapause elimination the rate of the process gradually diminishes as the temperature falls below optimum, and the rate falls off more abruptly if the temperature is raised. It is the similarity between the two curves that most strongly suggests that diapause breaking is also a form of development (cf. (1)). This diapause development must be completed before the more rapid postdiapause development and metamorphosis can begin.

Sometimes the rate–temperature curves for the two processes overlap extensively, sometimes not. An extreme overlap is evident where Matthée (28) found that diapause in *Locustana* was eliminated at 35° C. Another is evident in Dickson's (10) work on *Grapholitha*, which showed diapause development to be more rapid at 26° C. than at lower temperatures. In *Cephus*, on the other hand, the optimum for morphogenesis is about 25° C. and for diapause development, 10° C. (33).

Diapause and Its Study in Cephus cinctus

The experiments with which this paper is concerned were intended to discover whether there is evidence to support the hormone failure theory of diapause in the wheat stem sawfly, *Cephus cinctus* Nort., and whether the theory could profitably be used in interpreting this phase of the animal's life.

Because diapause in the sawfly is obligatory, the species does not lend itself to study of the original cause of diapause. It would be difficult to determine

whether and to what extent external conditions, such as declining temperature, progressive drying of the host plant, and diminishing photoperiod, are in fact the forces that commit the larva to dormancy upon its maturity since they are invariably effective in doing this in any naturally occurring situation. For this reason no attempt was made to study that aspect of diapause.

On the other hand, there are some advantages in knowing that every mature S-larva enters diapause. Furthermore, diapause in *C. cinctus* is a well defined state, yet it never outlasts more than one winter or an equivalent measure of laboratory chilling (33). The response of diapause larvae is well differentiated from that of other stages and one can be sure that a specimen kept at room temperature will not sometime emerge from diapause. These characteristics were found to be of advantage when manipulating the insects for experiments on development and emergence from dormancy. A frequent source of frustration was the "reluctance" with which postdiapause larvae began to develop when taken from the stubs and cocoons, where they normally overwinter and pupate, for treatment (7), but this in itself eventually proved to be informative.

Of interest is the larva's apparently unique capacity for returning to diapause when it has just gone through one such stage (7). Although *C. cinctus* is poorly suited to a study of the establishing of its fall diapause, it does provide an opportunity to study the establishing of a second, "spring" diapause.

Materials and General Methods

Sawfly larvae were collected, conditioned, and stored in the same way as those used in the earlier work on moisture conditions and diapause (7). Experimental material was thus prepared that was known to be in diapause, postdiapause but otherwise undeveloped, or postdiapause and partially developed—as the material was needed. As before, temperatures were maintained within $\pm 1°$ C. at 30° C. or over, and within $\pm 0.5°$ C. at 25° C. or less. The same test as before was applied for the presence of diapause, i.e., if larvae did not develop when incubated a month or more at 25° C. in their stubs then they were presumed to be in diapause. Since 25° C. is about optimum for postdiapause development all incubation was done at that temperature. The rate of development at 25° C. was taken as standard and all references to S-larval or prepupal age are based on it.

For ligation experiments performed to locate an active endocrine center, larvae were removed from their wheat stubs and ligatures of fine silk thread dipped in molten paraffin and beeswax were tied tightly around them in appropriate places. A single knot was adequate because the wax held it firmly enough to prevent seepage of fluids from one section to another. In ligated insects most organs thought to possess an endocrine function, e.g., the supra- and sub-esophageal ganglia and associated structures, the corpora allata, and the corpora cardiaca, were contained in the head section. Isolated prothoraxes included nearly all of the prothoracic glands, and abdominal sections the gonads.

Ligated larvae, pairs of larvae in parabiosis, and larvae that had received brain implants were incubated at 25° C. in wax cells lined with blotting paper. Slot-shaped cells were melted into blocks of household paraffin wax, each quarter-pound block holding 25 slots. Each cell was 5 mm. deep and just wide and long enough to accommodate a sawfly or pair of joined sawflies. It was lined with a small folded rectangle of blotter. After an operated larva was inserted, one end of the blotter was folded over and pressed in around the insect. The blotter was moistened every three days, being allowed to dry out for a short time between wettings. Incubation was for two or three weeks and the final data were based on the numbers of insects still alive at that time. This rearing technique did not completely solve the problem of inducing postdiapause larvae to develop when extracted from their stubs, but enough developed when reared this way so that useful data could be obtained.

A standardized cytological method was employed throughout a survey of endocrine activity in the larval prothoracic glands. Larvae were cut in two and fixed in Bouin's fluid, run through an ethanol series to Terpineol, and embedded in Tissuemat. They were sectioned at 6μ and stained with Heidenhain's iron hematoxylin and eosin Y. At first two other fixatives and two other staining procedures were also tried in a variety of combinations. A modified Lane's fixative often shrank the cells badly, and Flemming's (with or without extra acetic acid) did not permit good differentiation of secretory inclusions. Neither Krichesky's modification of Mallory's aniline blue collagen stain nor Van Gieson's connective tissue stain differentiated both nuclei and inclusions very satisfactorily. However, all combinations did show essentially the same features in the glands' cycle of activity.

For searching out and inspecting neurosecretory cells in the brain a more specific technique was required. Gomori's chromium hematoxylin–phloxin stain was used. The stain differentiates neurosecretory inclusions exceedingly well and has been recommended by a number of authors. Dissected brains were fixed in Bouin's fluid, embedded in Tissuemat, and cut at 4μ. Thereupon the schedule of Gomori (16) was followed save for slight alterations in timing.

Control of Postdiapause Morphogenesis by the Prothoracic Glands

In the first part of this investigation three things were sought. The results would determine the course of other experiments. The information required was (*a*) whether *C. cinctus* larvae have prothoracic glands, (*b*) whether the activity of these glands initiates pupation in mature S-larvae, and (*c*) whether an inactivation of the glands causes diapause, which occurs between maturation of the larvae in early fall and their metamorphosis in the spring. Are there prothoracic glands in this species that secrete a growth and differentiation hormone soon after diapause development is finished, and will that hormone stimulate pupation in diapause larvae if it is artificially supplied?

Ligation and Parabiosis Experiments

Sawfly S-larvae from which chilling had eliminated diapause were ligated just behind the prothorax, mesothorax, or metathorax. They were incubated in wax cells at 25° C. as already described and were examined periodically. The chilling had lasted long enough so that most larvae had passed through the transition period and were ready to begin postdiapause development promptly. Another series was first incubated in their stubs for three days at 25° C. and then ligated the same as the others. The exact position of the ligature proved to be inconsequential, and in Table I the three groups in each series are combined.

TABLE I

Metamorphosis of postdiapause larvae of *C. cinctus* ligated between the prothorax and abdomen

Number of days preliminary incubation at 25° C.	Total number ligated	Total number surviving	Number of insects in which the following parts developed:		
			Anterior	Posterior	Neither
0	75	54	28	0	26
3	75	69	46	32*	23

** 18 retarded.*

In the first and less advanced series none of the isolated abdomens began development, whereas the anterior portions developed in more than half the specimens. In the older S-larvae of the second series many abdomens underwent metamorphosis despite the constriction, though some developed later than the anterior parts. Abdomens in which development was slower than the thoraxes are described as "retarded" in Table I. There must be an anterior center from which metamorphosis is stimulated. Moreover, there is a critical point beyond which the impetus furnished by the center is no longer a requisite for development. In many of the older specimens this critical point had passed. Since results are similar whether ligatures are applied just behind the prothorax or further back, the stimulatory center is probably in the head or prothorax.

Controls were incubated in their stubs. Approximately one third of them became prepupal between the fourth and fifth day, and another one half between the fifth and sixth, leaving only one sixth of them still S-larval. In the treated series the abdomens of one third to one half of the larvae were capable of development when ligated on the third day. Roughly the same proportion of them would have been prepupae two days later had they not been constricted. The critical point for the pupation stimulus may be estimated as about two days before the prepupal transformation.

It remained to locate the stimulatory center more accurately and to confirm the time of its activity. Larvae at exactly the same stage as those used before

were each ligated in two places: (*a*) between the head and prothorax, and (*b*) between the metathorax and abdomen. Others were similarly treated but were first permitted to develop in their stubs at 25° C. for 2, 3, and 4 days.

The majority of larvae ligated on the fourth day exhibited some development of all their parts. In six of the group constricted on the third day only the thorax developed, while the abdomen and head underwent no change from the original S-larval form (Fig. 1). In other specimens in this 3-day group in which all three parts eventually did develop, development in most of the abdomens and heads lagged behind that of the thorax by a few days. Few larvae that were ligated earlier than the third day of incubation developed at all. It is evident that the source of the stimulus to pupal differentiation is the thorax, not the head. Complete results are presented in Table II.

TABLE II

METAMORPHOSIS OF POSTDIAPAUSE LARVAE LIGATED BEHIND THE HEAD AND METATHORAX

Number of days preliminary incubation at 25° C.	Total number ligated	Total number surviving	Number in which the following sections developed:			
			Head	Thorax	Abdomen	None
0	25	19	0	2	2*	17
2	25	16	0	3	0	13
3	25	22	10†	17	11†	5
4	25	20	13	15	13	5

* *Retarded.*
† *Nine and eight retarded, respectively.*

About half the insects passed the critical point between the second and third day, and two thirds had passed it by the fourth. It may be recalled that in the controls one third became prepupal between the fourth and fifth day, and five sixths were prepupal by the sixth day. Comparison of the two indicated that the crisis does occur, on the average, about two days before the prepupal transformation, as previously suggested. Later experiments showed that in other batches of larvae the crisis generally occurred later, about one day before the change to prepupa.

Anterior sections of ligated larvae, especially isolated heads, did not survive as well as more posterior ones, perhaps because of an oxygen shortage. Yet, frequently, a developing thorax or even a head lived long enough to become a mature pupal section. Compound eyes became pigmented, mouth parts, legs, and wing buds formed, and the larval exuvium was loosened, though it could not be shed. Sometimes pigmentation of the pupal integument beneath the exuvium occurred. Other visible changes employed in deciding whether or not a section of insect had developed were the formation of optic lobes on

the brain, straightening out of the abdomen, the fine crinkling or pebbling of the integument that indicated the digestion of its inner layers preparatory to molting, and the digestion of the fat body, all of which are prepupal characters. The external appearance of a prepupa can be compared with that of a mature S-larva from Figs. 2 and 3.

The first experiment described above showed that the stimulus must be derived from the head or prothorax, and the second showed that it comes from the pro-, meso-, or meta-thorax. Its source, therefore, must be the prothorax, since this is the only segment common to the two regions found to be active. More operations were performed to confirm this deduction.

Larvae presumed to be near the critical point were ligated behind the head and at the anterior margin of the mesothorax. To eliminate the possibility of hormone seepage from the brain into the prothorax the head was cut off in front of the first ligature. Most of the isolated prothoraxes soon died. Nevertheless, in addition to several specimens in which positive results were questionable, five prothoraxes did begin to molt while the attached posterior sections remained unchanged. Successful results were also achieved in at least five other specimens in which a ligature was applied just behind the prothorax and the anterior half of the head cauterized to destroy the brain. The factor that stimulates metamorphosis after diapause, therefore, must certainly come from the prothorax.

The idea that the stimulus is a hormone diffused in the hemolymph was investigated next. Larvae were joined in pairs tail to tail, so that the two blood supplies were confluent. The tips of the abdomens of two larvae in different stages of development were severed and the ends of a glass microtube thrust into the wounds. Ligatures were placed just in front of the wounds, binding the larvae to the tube. In 10 parabiotic pairs in which developing prepupae were joined to diapause S-larvae, the blood from the prepupa caused the S-larva to commence pupal development. Most likely a hormone in the prepupal blood was responsible. Over 30 similar pairs lived at least two weeks but the S-larva did not develop. The surgery and the possibility of some prepupae being unable to provide enough hormone for a pair of insects would account for their failure. A large number of controls in which both larvae were in diapause proved that the surgical treatment alone was never effective in breaking dormancy.

That a hormone produced in the prothorax makes postdiapause morphogenesis possible in *C. cinctus* seems, therefore, well established. If its origin and effect can be considered sufficient evidence, one may conclude that it is the equivalent of the growth and differentiation hormone (GDH) of other insects.

The relationship between the GDH and diapause that Williams (52-54, 57) demonstrated in *Platysamia* pupae can, with reasonable certainty, be assumed to exist in wheat stem sawfly larvae also. Essentially all that restrains the morphogenesis of a diapause sawfly is lack of GDH. If the hormone is artificially supplied to diapausing tissues they are competent to develop. The last statement may best be taken with some reservation, though it seems

to be true in its main implications. Metamorphosis can be induced artificially at any stage of diapause but it seems easier if the subject is part way through it.

Parabiotic experiments with *C. cinctus* gave the impression that a stronger stimulus is required by larvae in fall diapause than it is in larvae in which a second diapause has been established right after the first. The latter insects probably were still not completely readjusted to dormancy and retained some of the advantage of their original diapause development.

According to Grison (17) *Leptinotarsa* reacts in a way that may be comparable. Active brains are less effective when implanted into adults that have just entered diapause than in adults two months more advanced but still definitely in diapause.

Examination of the Prothoracic Glands

Evidence was still meager that the pupation stimulus in *C. cinctus* is the prothoracic gland's growth and differentiation hormone; but if prothoracic glands were found and if their activity was correlated with pupation, then certainly that would be sufficient. Holmes (21) had just found paired strands of tissue in the sawfly larva's prothorax that resembled the descriptions of prothoracic glands in other insects. Cytological examination of these organs would show whether they were glandular and, probably, when they were active. The latter information would be particularly interesting when compared with the critical point determined earlier by ligation of the insects. Accordingly, more than 300 *C. cinctus* larvae in various stages were fixed, sectioned, and stained as in the procedure already outlined.

The suspicious strands of tissue did prove to be prothoracic glands. On either side of the insect the gland extends along the lower of two lateral tracheal trunks from inside the head capsule back almost to the first thoracic spiracle. Throughout most of its length it is closely appressed to the trachea and is generally only a single layer of cells. At the neck a thicker branch leaves the trachea and extends ventrolaterally to the body wall. The prothoracic glands of *C. cinctus* resemble more closely those of many Lepidoptera than those of *Apis* as described by L'Hélias (20).

The glands can be readily recognized in prepared sections but in dissections vitally stained with methylene blue or neutral red they cannot be surely defined. They merge with thick agglomerations of cells surrounding the tracheae where these join the spiracles. Just before the S-larva re-forms into the prepupa there is a proliferation and growth of the tracheal epithelium, and at this time the gland is nearly lost among the epithelial cells.

During development the glands exhibit striking cellular changes. That these changes are closely linked with metamorphosis may be realized from the descriptions that follow.

The Prothoracic Glands During Diapause Development

Larvae in three different stages of diapause development were examined. The first had received no chilling and therefore had not begun diapause

development, the second had been at 10° C. for one month, and the third had been at 10° C. for three months. The last group was presumably near the end of diapause but had not yet completed it. The structure of the glands was the same in all three groups. Throughout diapause the glands are obviously inactive. The nuclei are small and flaccid. They do not stain very darkly and contain none of the granular inclusions that signify the onset of endocrine activity. The cytoplasm is weak-staining, contains no inclusions, and is riddled with many very large vacuoles (Fig. 4).

The Prothoracic Glands During Postdiapause Development

Larvae collected early in the spring were conditioned at 0° C. so that postdiapause development would begin promptly and uniformly as possible. This material was then put at 25° C. and samples were fixed each day. Of the descriptions below, those pertaining to the last four days of S-larval life and the prepupa are based on this series. Other, less advanced but less uniform material had to be used for the earlier stage (age, prepupal minus 10–14 days).

Several representative stages were re-examined in material from other sources and gathered in another season. The behavior pattern determined for the glands was confirmed.

1. S-larvae Due to Become Prepupae in 10 to 14 Days (Aged Prepupal Minus 10–14 days)

The prothoracic glands appear more turgid than in diapause insects. Nuclei are fairly large and round, but still clear, except in the rare specimen where a few nuclei are rather dark. There are no inclusions in the cytoplasm, and vacuoles are still numerous.

2. S-larvae Aged Prepupal Minus 3–4 Days

Glands are turgid, nuclei large and rounded. Throughout most of the gland the nuclei are clear and have no notable inclusion granules. Cytoplasm is clear and light (Fig. 5). However, in the loose strand of tissue stretching from the trachea to the body wall some cells are beginning to show signs of hormone production: nuclei dark with granular inclusions occurring especially at periphery, cytoplasm still very clear (Fig. 6). Activity apparently begins at the lower anterior part of the loose strand, later spreads to the rest of the loose strand and from there to the posterior part of the gland.

The beginning of activity was apparent in nearly all specimens and in some was slightly more advanced. Thus the above description applies best to S-larvae about four days before the prepupal stage.

3. S-larvae Aged Prepupal Minus 2–3 Days

Nuclei are generally dark, filled with small, densely staining, granular inclusions. In the cytoplasm of many cells in nearly all specimens numerous small granules have appeared, first showing up around the nucleus. This change probably begins about three days before the prepupal transformation.

PLATE I

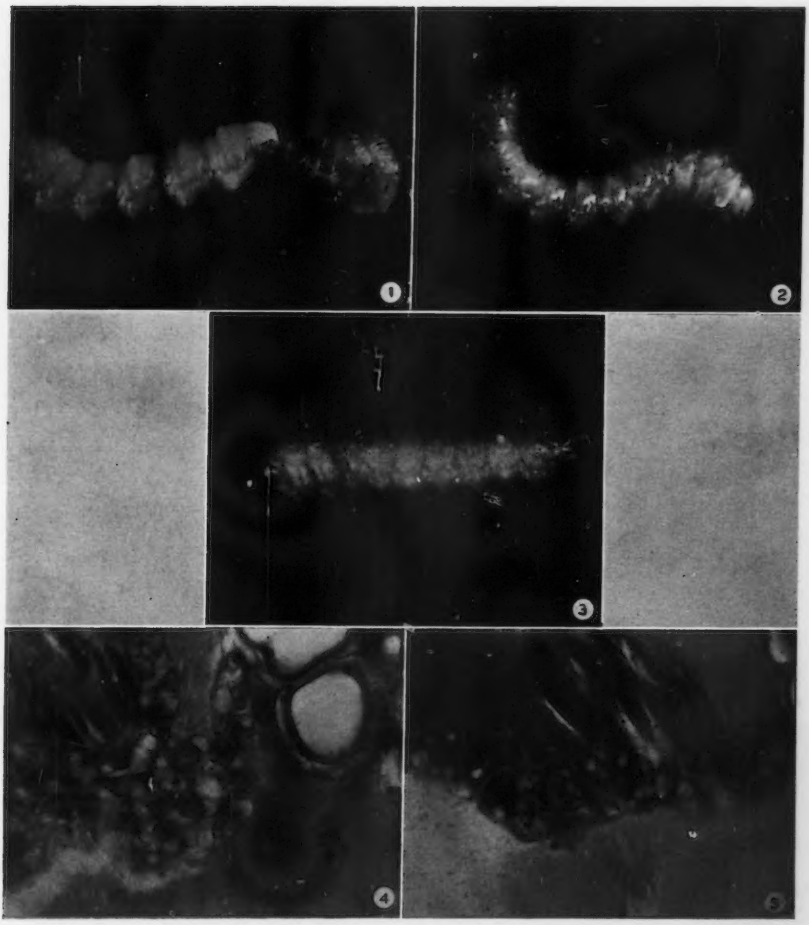

FIG. 1. *C. cinctus* S-larva ligated behind head and metathorax shortly before GDH crisis. Head and abdomen retaining their S-larval form; thorax developed to pupal stage (note patches of dark pigment under larval exuvium of the thorax). ×5.

FIG. 2. *C. cinctus* S-larva. Body characteristically curved, skin smooth. ×5.

FIG. 3. *C. cinctus* prepupa. Body straight, skin finely wrinkled, compound eyes formed and colored. ×5.

FIG. 4. Section of loose strand of the prothoracic gland near its junction with the trachea in a diapause S-larva. Cells lightly stained and vacuolated; nuclei small and flaccid. ×490.

FIG. 5. Section of loose strand of prothoracic gland. Postdiapause S-larva aged about prepupal minus 4 days. Cells lightly stained; nuclei fairly large and round. ×490.

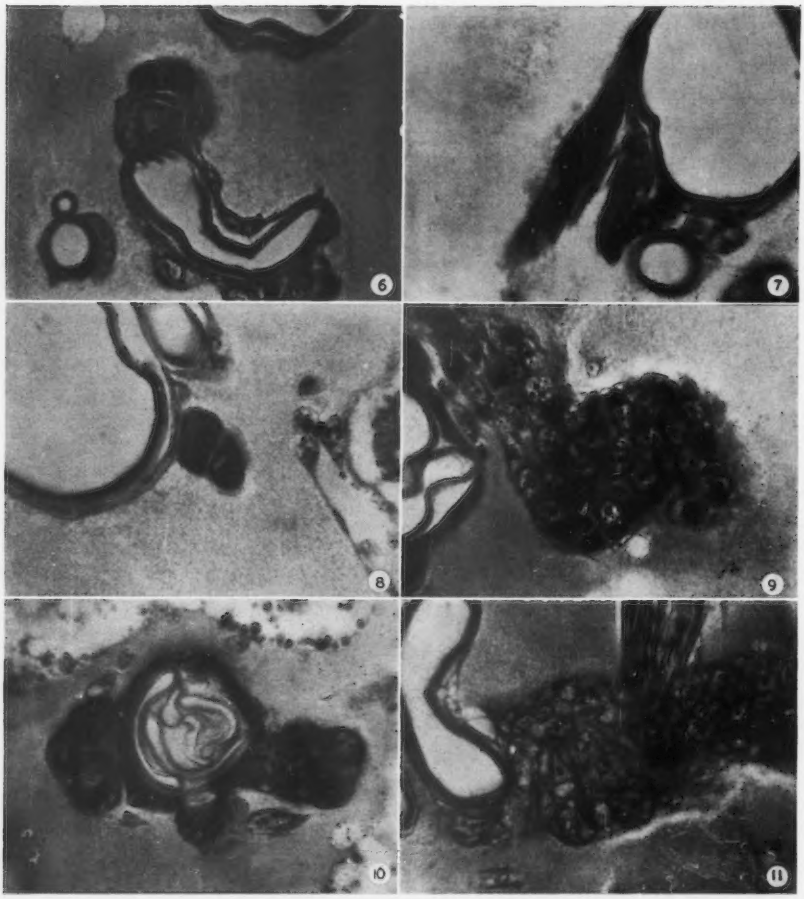

FIG. 6. Section of prothoracic gland cells attached to trachea. Postdiapause S-larva about prepupal minus 3 days. Some nuclei dark-staining. ×500. FIG. 7. Section of prothoracic gland at its junction with the trachea. S-larva about prepupal minus 2 days. Secretory material in nuclei and cytoplasm stained very dark. ×500. FIG. 8 Two prothoracic gland cells appressed to trachea. S-larva about prepupal minus 1 day. Some granular inclusions in nuclei; cytoplasm filled with dark-staining secretory material. ×500. FIG. 9. Section of prothoracic gland near its junction with the trachae. Prepupa about 2 days old. Nuclei and cytoplasm light-staining; large, dark inclusions in the cytoplasm. ×500. FIG. 10. Section of prothoracic gland cell on either side of trachea. Prepupa about 5 days old. Nearly all traces of inclusions gone. ×500. FIG. 11. Section of prothoracic gland near its junction with trachea. S-larva in which diapause has just been reinstated by a 7-day exposure to 35° C. Note close resemblance to Fig. 4. ×500.

In about half of the insects some of these granular cytoplasmic inclusions have apparently coalesced to form larger masses, which may then appear as moderately large droplets. This begins about two days before the prepupal stage is reached.

It is believed that the hormone is produced in the cell nucleus, from there passes into the cytoplasm, and thence soon flows into the blood. The appearance of sizable droplets in the cytoplasm probably indicates that the liberation of the growth and differentiation hormone has begun.

4. S-larvae Aged Prepupal Minus 1–2 Days

Hormone secretion is apparently in full swing. Most nuclei are very dark with many granules (Fig. 7), though scattered nuclei may be clear. Cytoplasm is generally dark with many granules and large, amorphous droplets or aggregations of granules. Some cells are completely dark.

5. S-larvae Aged Prepupal Minus 0–1 Day

Some nuclei are still dark and filled with granules, but most have now cleared somewhat. Cytoplasm contains large, dark, dense, amorphous drops of secretory material, some of them appearing to fill the entire cytoplasm (Fig. 8). Other cells have many smaller droplets.

6. Prepupae Less than 1 Day Old

Nuclei in many specimens are still rather dark. They have been growing increasingly large and rounded until this stage. Cytoplasm contains large, dark, amorphous drops; the drops are more compact and well-defined than previously.

7. Prepupae 1–2 Days Old

Nuclei are clear; cytoplasm is clear but contains large drops, typically one drop per cell, about two thirds the size of the nucleus and very clearly defined (Fig. 9). The cells have probably ceased endocrine activity. Except for the presence of drops in the cytoplasm they appear quite inactive; certainly the nuclei have stopped producing new material.

8. Prepupae 2–4 Days Old

In some cells the cytoplasmic drops are noticeably reduced in size and appear as crescents applied to the outside of the nucleus.

9. Prepupae 4–5 Days Old

Cytoplasmic drops appear only as small shadows.

10. Prepupae 5–6 Days Old, Ready to Pupate

Both cytoplasm and nuclei are completely clear (Fig. 10).

In summary, the prothoracic glands appear not to be directly concerned with diapause development, no apparent changes in them occurring during that period. They are activated only after diapause is over. Signs of

activity appear at least four days before the transformation to prepupa, and the glands probably begin releasing the GDH into the blood at least two days before the prepupal stage. They continue to secrete for about the next four days. Ligation experiments with specimens from this stock of sawfly larvae showed that a critical amount of GDH had accumulated in the blood a day before the prepupal stage, i.e., about one day after its secretion began. At that point if the head or abdomen were isolated from the thorax its metamorphosis would not be stopped. The extra hormone released after that might be regarded as providing a safety margin.

Extent of Control Over Imaginal Differentiation Exerted by the GDH

Information that shows imaginal development does not come to a dead halt during diapause is becoming increasingly common. The survey of the prothoracic glands incidentally furnished an additional example of this. The cell masses in the brain that ultimately form adult structures and the definitive imaginal legs and wings enlarge throughout the dormant period, though only slowly. Typically they may double in sectional diameter during diapause, before the GDH is released. They must be independent of the GDH up to a point, but they could never mature without it.

The same principle is well illustrated by some work done in collaboration with Margaret Rowlatt Mackay on the larval testes of *C. cinctus*. Spermatogonial mitosis is not interrupted by diapause. Rather, it runs parallel to diapause development, proceeding more rapidly at 10° C. than at either warmer or cooler temperatures, though cell divisions do continue even at 25° C. However, the GDH must be available before the cells can differentiate, before meiosis and spermatid formation can occur. If the GDH is not supplied opportunely, any spermatocytes already formed in the testes degenerate and are sloughed off, but they are continually being replaced by new ones produced from less advanced spermatogonia.

Schneider (40, 41) has reported extensive growth and maturation of imaginal tissues in dormant syrphid larvae. In some species of Syrphidae imaginal structures are sufficiently mature by the time the larva is fully fed so that it can pupate without delay. In other species the imaginal organs lag far behind in their growth and these insects enter diapause at the end of the larval stage. Their imaginal organs grow fairly rapidly at low temperatures and are "mature" when diapause is over. Indeed, Schneider attributes diapause to the inhibition of growth of the imaginal anlagen and their resulting "maturation deficit", and thinks that it cannot end until the organs attain normal maturity.

Of equal interest are the reactions of the imaginal tissues and the processes of metamorphosis when the prothoracic glands begin to secrete after diapause. When a certain minimum concentration of GDH in the blood has been attained the imaginal tissues and organs responsible for molting will respond. But it seems that more hormone is needed to sustain the processes that it started.

Some may be needed merely to replenish what breaks down, but also a higher concentration of hormone may be required if development is to continue beyond its most primary stages. At any rate the processes of molting and metamorphosis do not immediately lose their dependence upon the GDH, but require its continued presence for a time after morphogenesis has begun. So far this is known to be true of *Drosophila* (46) and *Platysamia* (22).

In *C. cinctus*, too, there seems to be an interval after metamorphosis has begun when interruption of the flow of GDH will stop development. If postdiapause larvae are removed from their stubs and cocoons after the critical point in the GDH secretion cycle, or at least after it is estimated to have occurred, nearly all of them pupate. Fewer develop if taken from the stubs a short while before the crisis, and when extracted several days before many of them sometimes will not develop though they live for months, seemingly healthy (7). The reason for the developmental failure may be that the GDH is not secreted, or its secretion is interrupted. The results of an examination of prothoracic glands in larvae whose metamorphosis was thus inhibited support such an idea. Some glands began to secrete, or at least manufacture, GDH but did not progress very fast or far.

In certain specimens the crisis comes much later than the time estimated for typical larvae. Occasionally even a very young (but unmistakable) prepupa will develop no further when taken out of the stub. It must have had sufficient GDH to begin metamorphosis but not enough to carry on. Very young prepupae, unlike the more advanced ones, often stopped developing or developed incompletely when joined in parabiosis to diapause insects. In no such case did their diapause partners develop either.

These observations indicate that the continued secretion of GDH is necessary in *C. cinctus* until development is well established. Usually it is dispensible one or even two days before the prepupal transformation, but sometimes not until after the prepupa has formed. Further, the observations presuppose that the production of GDH may be hampered if developing S-larvae or young prepupae are disturbed. Perhaps the prothoracic glands are under the influence of the brain and nervous system and remain so until well into their secretion cycle.

Influence of the Brain on the Prothoracic Glands

The control exerted by the prothoracic glands over postdiapause morphogenesis in *C. cinctus* has been fairly well established. But one must go back, several steps perhaps, along the course of the insect's development before one reaches the diapause stage itself. This section concerns the first step back, where attempts were made to determine what controls the prothoracic glands, what impels them to activity after their long rest. It was related in the introduction that the brain is known to function thus in a few insect species. Therefore it seemed reasonable to concentrate upon the brain in *C. cinctus* and the possibility that it produces a hormone that acts upon the prothoracic glands.

Ligation and Implantation Exeriments

Thirty-five larvae were constricted close behind the head when prepupal minus 5–6 days of age. Another 35 were incubated in their stubs for three days until they were prepupal minus 2–3 days of age and then were similarly ligated. Controls of the same ages were ligated behind the thorax. In either age group so long as there was no constriction between the head and prothorax the thorax more often than not was able to develop. Despite constriction behind the head in the prepupal minus 2-3 day group the thorax was able to develop in some larvae. Ligating off the head in the prepupal minus 5-6 day group, however, blocked development in all specimens (Table III).

TABLE III

DEVELOPMENT OF POSTDIAPAUSE S-LARVAE LIGATED

Age when ligated	Where ligated	Number surviving	Number in which thorax developed
Prepupal minus 5–6 days	Behind head	23	0
	Behind thorax	31	17
Prepupal minus 2–3 days	Behind head	30	7
	Behind thorax	25	20

Essentially similar experiments were repeated many times. Postdiapause S-larvae were operated upon at various stages of development. Some were in the first part of their transition period and others as much as a couple of weeks at 25° C. more advanced, by which time they were about five days from becoming prepupae. Even when not deprived of the function of their brains many such larvae did not develop when taken from their stubs. When the head was removed or the brain destroyed by cautery none of them developed at all. When the S-larvae were as close as three days to the change to prepupa destruction of the brain removal of the head reduced the chances of developing from perhaps 70% to 10%. It was judged from sections and dissections of operated specimens that treatment affected only endocrine organs within the head. Cautery of the front of the head and the brain did no visible damage to the prothoracic glands, and ligation seldom injured more than a small portion of the anterior parts of them.

These experiments, considered by themselves, seem reasonably convincing. It would appear that the brain or some organ closely associated with it in the head produces a stimulus that activates the prothoracic glands. Typically it seems to be produced in an effective amount two or three days before the prepupal stage. The exact time of the critical point appears very variable; it can be earlier and is often later. After this crisis in the brain's activity the prothoracic glands become dissociated from its influence and shortly after reach a crisis of their own, in turn becoming dispensible.

Other experiments done in conjunction with the preceding ones showed the real situation to be more complex than proposed in the last paragraph. In

all experiments taken together the thorax developed in two thirds of the specimens ligated behind the metathorax if the head was removed or the brain cauterized as well. The thorax developed in less than half of those that were just ligated once, behind the metathorax. Loss of the brain evidently stimulated metamorphosis of the isolated anterior section. The experimental material was from various sources and ranged in age from prepupal minus 4 days to prepupal minus 1 day.

In order to corroborate this observation, S-larvae aged prepupal minus 8–12 days and prepupal minus 0–3 days were operated upon. Fifty of each age were constricted behind the thorax and their brains destroyed with a hot needle, 50 were just constricted behind the thorax, 50 were just cauterized, and another 50 were not operated upon. All were then incubated in wax cells as usual. The results are shown in Table IV. As expected, the thorax most frequently developed in the specimens where the two operations were combined. By itself, destruction of the brain hindered development as it had in other experiments.

TABLE IV

STIMULATION OF THORACIC DEVELOPMENT BY COMBINED LIGATION AND BRAIN CAUTERY

Treatment	Age when treated	Number in which: Thorax developed	Thorax did not develop
Untreated controls	Prepupal minus 8–12 days	10	36
	Prepupal minus 0– 3 days	13	30
Brain cauterized	Prepupal minus 8–12 days	0	41
	Prepupal minus 0– 3 days	1	40
Ligated behind thorax	Prepupal minus 8–12 days	2	42
	Prepupal minus 0– 3 days	8	39
Ligated behind thorax and brain cauterized	Prepupal minus 8–12 days	13	26
	Prepupal minus 0– 3 days	22	21

The experiment was repeated another season with prepupal minus 1–3 day larvae. Results were practically the same. This time a ligature behind the thorax stimulated thoracic development, but, again, there was a much greater effect when one behind the head was added to it.

No explanation can be offered for this unusual effect. A ligature behind the thorax would confine all the GDH to the front of the insect, and that could be critical in cases where too little was produced to initiate morphogenesis in the whole animal. But why should loss of the head or the brain and neighboring tissues further stimulate development of the thorax when it is separated from the abdomen, yet hinder that development when thorax and abdomen are left in union? There seems to be no simple answer.

Previously it was proposed that in *C. cinctus* the prothoracic glands may be activated by a simple, direct stimulus from the brain. The experiments just described, however, weigh against the suggestion. Sometimes destruction of the brain (or better, the operations by which the brain, among other things, is destroyed) inhibits development, and sometimes promotes it. Certainly, more is involved than a *simple* stimulation of the prothoracic glands. An interaction between the brain and the prothoracic glands is indicated, although up to this point its nature has remained elusive.

More information was sought by different means. Brains were dissected in Ringer's saline from postdiapause S-larvae of prepupal minus 7–11 days of age. Three brains were implanted into the abdomen of each host larva through an incision at the abdominal tip which was then closed with a ligature. The hosts were diapause S-larvae that had received no chilling. No rigorous sterile technique was employed, but care was taken to keep all materials and animals clean. Twenty-one out of 103 experimental specimens lived for more than three weeks. Of these, two became prepupae in about two weeks. Seventy-nine controls that had received an injection of Ringer's but no brain implants lived at least three weeks, but none developed. The results are meager but significant; there can be little doubt that the two larvae that began morphogenesis were made to do so by the implanted brains. The brain, apart from its associated organs, evidently is able to activate the prothoracic glands. All connective nerves from the brain were broken when the implants were made. Therefore the operating medium of the successful brains was most likely a hormone.

Hormone secretion may not be the only function of the brain in this regard, but it would seem to be of some importance. Actual evidence of endocrine activity by the brain would give this conclusion a better foundation. It was thought that such evidence might be gotten from a cytological study of the brain cells.

Survey of Neurosecretory Cells in the Brain

Brains from a progression of sawfly larvae were removed, fixed, sectioned, and stained with Gomori's chromium hexatoxylin – phloxin. In all, well over 100 brains were examined. Brain cells in *C. cinctus* are so very small that even under very high magnification little detail is seen. Nevertheless, because of the excellent differentiation of neurosecretory inclusions that can be achieved with Gomori's stain, the survey was reasonably successful.

Traces of neurosecretory activity were found to occur at all stages investigated and many parts of the brain had one or two secretory cells. Several active cells were often found in the ventral region of each hemisphere but they formed no discrete body and their activity seemed irregular. Two conspicuous neurosecretory cell aggregations occur in the dorsal part of each hemisphere and each forms a fairly compact mass. They are obviously the cell groups that commonly are to be seen in the insect protocerebrum and that have been linked with the reactivation of *Platysamia* at the end of diapause

(55). A medial group extends several cell layers inwards from the hemisphere's dorsal surface. It is near the center of the brain and usually merges with its counterpart on the other side. In one hemisphere the group may contain as many as 20 active cells. The other cell mass also extends inwards from the dorsal surface of the hemisphere but is nearer the lateral edge. It is smaller, and 15 active cells were the most seen in one group. The position and appearance of the two pairs of cell masses are very similar to those of *Lophyrus* as described and illustrated by L'Hélias (19).

Though larger than most other brain cells in *C. cinctus*, the neurosecretory cells were mainly distinguished by the great numbers of small, round droplets that were often contained in the cytoplasm. These inclusions were the only structures that stained an intense black with Gomori's stain, a distinguishing characteristic of neurosecretory material.. Both the medial and lateral groups exhibited a progressive increase and decrease in the number and size of secretory inclusions as the insects developed. The changes are summarized in Table V.

The time of most intense activity did not coincide for the medial and lateral groups, occurring later in the latter. The inner groups began to increase markedly in activity before the end of diapause, and reached a peak during the transition phase. When larvae were still a week or two from becoming prepupal, secretory activity began to decline. As the prepupal stage drew near, the cells became nearly bereft of inclusions. Cells of the lateral groups had some inclusions even during the early part of dormancy and there was no sizable increase until the prepupal stage was about a week away. The rise and fall of activity in the lateral cells was much less conspicuous than in the medial ones.

The more intensive activity of the medial neurosecretory cells and the fact that it is concentrated into a sharper peak suggests that they are the more important. But the timing of the activity of either the medial or lateral cells fits in with the idea that the brain initiates postdiapause morphogenesis, and that it does this in part by endocrine action, its neurohormone presumably stimulating the production of GDH by the prothoracic glands.

The peak of the brain's activity, as judged from the presence of inclusions in the neurosecretory cells, occurs at least a week, perhaps two or more, before the activation of the prothoracic glands is complete. After the neurohormone is seen in abundance in the cell cytoplasm a long interval elapses before it can finally have accomplished its effect. The interval must be an important one but we do not know what happens during its passage.

It is tempting to think that a specific amount of neurohormone is required to activate the prothoracic glands in an insect, that before this crucial quantity has been released the glands are wholly inactive, and that once the right quantity has been produced the glands are activated (or will be as soon as it takes effect), and from then on have no further demands upon the neurosecretory cells. But this cannot be true. In *C. cinctus* noticeable changes take place in the prothoracic gland cells at least one day, but more commonly

TABLE V

RELATIVE ABUNDANCE OF NEUROSECRETORY INCLUSIONS IN THE BRAINS OF MATURE S-LARVAE AT DIFFERENT PHASES OF DEVELOPMENT

Stage of development of larvae	Medial group of neurosecretory cells	Lateral group of neurosecretory cells
In early diapause (kept at 25° C.)	A few small inclusion droplets in a few of the cells	Occasionally some fairly large droplets in a few cells
In late diapause and early transition period (half the insects in the sample were the latter, due to become prepupal in 20–28 days at 25° C.)	Inclusion droplets in most cells; numerous large ones in many of them	Occasionally a few small droplets in a few cells
Postdiapause, at 25° C. would become prepupae in:		
8–16 days	Many droplets, usually large ones, in most cells	Scarcely any inclusions present
6–14 days	Slightly fewer inclusions present than above	A few inclusions present
3–11 days	Inclusions still present in some cells; fewer droplets in each	Many fairly large droplets in some cells
0– 8 days	Inclusions in only a few cells; some droplets very shadowy	Inclusions in only a few cells; some droplets shadowy
0– 4 days	Inclusions in very few cells	Inclusions in very few cells

several, before the glands can carry on alone. Either they continue to depend upon the neurohormone for a time after their reactivation has begun, or the neurohormone by itself cannot always do the whole job—or, of course, both.

The presence of inclusions in the neurosecretory cells is not necessarily a sign that free neurohormone is shortly to appear in the blood or in some other way be made available to the prothoracic glands. It may not be released to act upon the glands until days later. There is some possibility that it travels from the cell bodies along nerve fibers to the prothoracic glands. If so, the trip might take some time.

Protocerebral neurosecretory cells in insects are functionally related to two endocrine systems, the cardiacum–allatum system (2, 19, 36, 45), as well as the prothoracic glands (48, 51, 53, 55). Moreover, judging from the descriptions in the literature just mentioned, the same cell groups are involved. The secretion from the neurosecretory cells normally follows the axons originating from the cells into the corpora cardiacum–allatum complex. Arvy and Gabe (2) found in some Plecoptera that a pair of nerves continue on from the corpora allata into the prothorax. These nerves also carry neurosecretory material. Do they by some chance lead to the prothoracic glands and do they convey some of the neurohormone there? From the standpoint of conserving the little neurohormone that the insect produces it would be advantageous to confine it to the parts where it is useful. Cutting the connectives from the brain has been demonstrated to interfere with its activity. In *Calliphora* implantation of brains and ring glands (whose lateral cells are homologues of the prothoracic glands) with the connectives intact between the two was shown to be more likely to effect metamorphosis than implantation of the same organs with the connectives severed (29).

On the other hand it is also true that cutting their axons does not completely prevent the neurosecretory cells from functioning. This is evident from the fact that brains implanted without intact nervous connections have many times been demonstrated to be effective. (In this connection it may or may not be significant that many such brain implants, though viable, are ineffective even where several brains are used in one host.) Although it may normally follow nerve fibers, the neurohormone can obviously also be released into the blood and still accomplish its task.

Nervous links between ganglia and glands may well be important quite apart from the conveyance of neurohormone. There is much to suggest that the relationship of the brain to the prothoracic glands is not merely one of simple stimulation at the appropriate time, although the brain evidently does that, too, and it is sometimes sufficient. But in addition it seems to exert a limited control over the glands. This might involve nervous as well as endocrine elements and, of course, other organs besides the brain and prothoracic glands.

Most certainly, it is insufficient that the one neurohormone should simply activate both the cardiacum–allatum system and the prothoracic glands at the

same time or in close sequence, with neither the neurohormone nor other elements of the brain or other organs having any influence or control over the glands from then on. Else how could the balance between juvenile and imaginal development, not to mention diapause, be so delicately adjusted, emphasizing first one, then the other, thereby to produce the different phases of larval development, metamorphosis, and reproduction in their proper places?

Environmental Inhibition and Stimulation of Morphogenesis in Postdiapause Larvae

The "reluctance" of healthy and vigorous sawfly S-larvae to begin postdiapause development out of the stub is still a source of some puzzlement. It was especially so at first because inside the stub they are not particularly sensitive to adverse physical conditions. Desiccation will inhibit metamorphosis but only if it is severe (7). It was later realized that even out of the stub it is not such things as unfavorable moisture or light conditions that discourage development. Care in keeping the larvae moist, but not too moist, or in avoiding even the shortest exposure to light avails little. Thousands of larvae have been used in attempts to rear them out of the stubs under what would seem to be the most favorable of conditions; they live but the majority do not pupate unless they are already near the GDH crisis.

Actually, such treatments as short periods of drying alternated with wetting, short exposures to heat, and dipping in sulphuric acid often stimulate more of them to develop, but are never completely effective. Constant exposure to light is inhibitory. The rearing method used throughout this work—incubation in wax cells lined with blotting paper, where the paper is pressed closely around the insect and is kept alternately moist and dry—has for some reason proved the most productive. Thus handled, most develop if they are about three days from becoming prepupal. Other attempts to imitate the insects' natural habitat—wrapping them in paper, enclosing them in glass or cellophane tubes—were not very effective.

The stub in which the larva lives is lined with a continuous membranous cocoon, and really it is the crucial thing rather than the stub. A larva will pupate as readily in a cocoon extracted from the stub as it will in one left in place. The idea was once considered that the larva may depend upon a particular atmosphere created by the cocoon serving as a partial barrier to the diffusion of gases. This idea was abandoned when S-larvae were found to develop as well in broken or incomplete cocoons. They were reared in stubs that were split open and tied together again after the cocoons had been removed. They fashioned new cocoons of a sort, very thin and discontinuous, and then all pupated.

After consideration of the things mentioned above the most plausible explanation for the inhibitory effect of extraction from the cocoon may be that the "feel" of the larva's surrounding—their texture and shape—is partly

responsible for its behavior. Sensory stimuli received by the brain perhaps affect its messages, hormonal and nervous, to the prothoracic glands.

If interpreted in this way, the insect's behavior strengthens the view that the prothoracic glands are controlled by the brain. Because of the nature of the stimuli (the physical structure of the habitat, short periods of drying and wetting, quick dips in strong acid, short heat exposures) that can to some extent influence the initiation of postdiapause development, it seems less likely that they would affect the prothoracic glands directly than indirectly through the brain.

The response of *C. cinctus* to various stimuli may be comparable to the responses of some other species to "shock" treatments. Pricking, singeing, oviposition by a parasite, sharp temperature changes, and the action of acids and other chemicals have been reported in numerous papers to be effective in breaking diapause. As Andrewartha (1) has suggested, these in fact probably do no more than prompt a somewhat premature reactivation of insects with a "weak" diapause or of those nearing the end of a "stronger" one after having completed the main part of diapause development. Thus the sensitive stage in these species is little different from that of *C. cinctus*. The shocks presumably stimulate the nervous system. Once the stage is nearly set for the new phase of development, a strong stimulus will sometimes invoke the nervous–hormonal mechanism prematurely, causing development to begin immediately.

At least one species has to have a final stimulus at the end of diapause or it can go no further. *Locusta* eggs, even if reared during diapause at a constant temperature well above the threshold of development, will not start post-diapause development unless stimulated by a rise in temperature (3). It is worth recalling, too, that Wigglesworth (47) demonstrated that the brain in *Rhodnius* triggers the molting process in response to a stimulus provided by distension of the abdominal wall accompanying the ingestion of a large meal. In many other species outside stimuli are likely much less decisive or perhaps totally without effect.

Oftentimes the brain becomes insensitive to such stimuli at an early stage of the nervous–endocrine cycle. In many cases, even occasionally in *C. cinctus*, activated brains have become sufficiently independent before secreting the neurohormone so that they can continue to function even when excised and implanted in new hosts. Some experiments by Bounhiol (6) on *Bombyx* larvae illustrate this point very well. At a certain stage of development severance of the brain's nervous connections by its removal and reimplantation prohibits development. About 24 hours later, however, removal of the brain prevents development, but its immediate reimplantation allows development to proceed, despite the fact that all the brain's nervous connections with the rest of the body were severed as before.

In other instances, however, there is apparently much overlap of sensitivity, where the brain is still susceptible to influence by sensory stimuli long after neurosecretion has begun, and, as related earlier with regard to some *C. cinctus*

specimens, the prothoracic glands remain susceptible to influence by the brain when GDH secretion is well under way. Thus could a young sawfly prepupa be inhibited from further development when taken from its cocoon. The argument looks to be reasonable, though thin in places.

Reinstatement of Diapause

The ability of *C. cinctus* S-larvae to revert to diapause when warmed to 35° C. was demonstrated by Salt (33). S-larvae remain viable after several weeks at 35° C., exhibiting no outwardly visible development during that time, whereas prepupae become comatose and die. Salt's (34) experiments have also shown that diapause can be reinstated in some specimens at as low as 30° C. At that temperature some S-larvae will also develop into normal adults, whereas still others develop abnormally and eventually die.

Diapause is reinstated in the most susceptible members of a population in two days at 35° C., and six or eight days' exposure, opportunity made, is enough to affect all of them. The response of such larvae to chilling at 5° and 10° C. is identical with that of normal diapause larvae (7). Moreover, ligation and parabiosis experiments have shown that morphogenesis is resumed in response to the GDH just as in the case of fall diapause. Thus the reinstated, "spring" diapause closely resembles the original.

The effect of 40° C. is much weaker than that of 35° C. Repeated testing has proved this rather remarkable observation to be true. A typical example is shown in Table VI, the result of an experiment in which samples of about 30 larvae in the stub were kept at 35° and 40° C. in moist soil for 3, 5, and 7 days. They were incubated at 25° C. and the extent of diapause reinstatement was figured from the number of larvae that failed to develop. Five or seven days at 35° C. returned many more insects to diapause than did an equal period at 40° C. It should be mentioned that, although longer periods at 40° C. are lethal, seven days at 40° or at 35° C. did not cause any mortality.

TABLE VI

PERCENTAGES OF S-LARVAE IN WHICH DIAPAUSE WAS REINSTATED BY EXPOSURE TO 35° AND 40° C. FOR VARIOUS PERIODS

Length of exposure, days	Temperature	
	35° C.	40° C.
3	0	4
3	6	3
5	52	10
5	53	16
7	92	14
7	93	9

Forty-five degrees centigrade is lethal within about two days, and sublethal treatments arrest development. However, few larvae in which development is arrested by exposure to 45° C. can be in diapause because chilling does not reactivate them. A few specimens develop but most eventually die as S-larvae. This is in sharp contrast with the response of larvae treated at 35° C.

The problem remained of defining the stage during which sawfly larvae are capable of returning to diapause. For this purpose postdiapause S-larvae, conditioned at 0° C. to begin development promptly, were incubated at 25° C. Each day samples were removed from incubation and (*a*) four groups of about 25 larvae in their stubs were subjected to 35° C. for 7 days to reinstate diapause wherever possible, (*b*) a group of 25 were ligated behind the thorax to determine the number that had passed the crisis of GDH secretion, and (*c*) a group of about 50 larvae were examined to determine the number that had transformed into prepupae. The three series may be compared from the summary of results in Table VII.

TABLE VII

PERCENTAGES OF LARVAL POPULATION AT CERTAIN STAGES OF DEVELOPMENT AFTER INCUBATION IN THEIR STUBS AT 25° C. FOR VARIOUS PERIODS

Incubation in stubs, days	Percentage of population that:		
	Had passed susceptible stage for diapause reinstatement	Had passed GDH crisis	Had become prepupae
0	0	0	0
1	5	15	0
2	23	27	12
3	68	52	37
4	77	77	67
5	97	90	78
6	—	—	88
7	—	—	100

An adequate amount of GDH was secreted about a day before the prepupal transformation. The susceptible stage for diapause reinstatement ended about a day before the prepupal transformation also. Actually it was slightly less than one day in the larvae that transformed first, and a little more in the slower insects. It is concluded that the point where the ability to return to diapause is lost coincides reasonably closely with the secretion of an effective concentration of differentiation hormone.

If the GDH crisis has not yet passed, postdiapause S-larvae can revert to dormancy whether they have just emerged from diapause or are only a day or two from being prepupal. It does not matter whether they are heated before or after the transition stage. If they have not already begun postdiapause development, the first few hours or the first day at 35° C. forces them to the

point where they are ready to begin it without delay. For example, larvae just out of diapause, when subjected to 35° or 40° C. for a day or so and then incubated at 25° C., become prepupae in about 7 or 8 days. It would have taken them two or three times as long if they had been incubated at 25° C. unheated.

Examination of the prothoracic glands and brain during the process of diapause reinstatement suggested a mechanism that might possibly be the one whereby reinstatement is brought about. Stained sections were prepared as before from postdiapause larvae that had been warmed to 35° C. for 0, 2, 4, and 7 days, and 7 days followed by 4 days of incubation at 25° C. The week at 35° C. returned all specimens to diapause as planned.

At the beginning of the treatment the larvae were three to four days from being prepupae, and some of the lower anterior cells of the prothoracic glands had begun to show signs of activity. No change was apparent after just two days at 35° C. but thereafter the inclusions that were originally present gradually disappeared. Nuclei and cytoplasm became very clear and vacuoles formed. Finally, after 7 days at 35° plus 4 days at 25° C., the nuclei had lost some of their turgidity and the glands had come to resemble those of diapause larvae (Fig. 11). Of course, by this time that is just what the larvae were. GDH production and secretion was obviously stopped abruptly by the heat, not to be resumed, presumably, until after several months of chilling.

Examination of the brains from a parallel series of larvae was less informative. At the beginning of the treatment the surge of postdiapause neurosecretion was nearly over, and no noticeable changes took place in the neurosecretory cells while the insects were being heated. Unfortunately, lack of time prevented any more experiments.

When these few facts concerning diapause reinstatement are assembled, they might be interpreted in the following way. Heat prevents production of the GDH, or stops it abruptly if it has already begun. Meanwhile the stimulus to which the prothoracic glands have been responding is destroyed. There is evidence that at least part of this stimulus is provided by the neurohormone. It may gradually break down during the interval when the prothoracic glands are incapacitated. By the time the heat treatment is finally suspended there is too little neurohormone left to have any effect on the prothoracic glands and they revert to complete dormancy. Perhaps at 40° C. the prothoracic glands are temporarily inhibited as they are at 35° C., but the reactions whereby the neurohormone is dissipated are inhibited as well. The result is a state of "suspended animation" that the insects can endure for a week or more and which results in neither an alteration of the life cycle nor any permanent harm.

It is interesting that the growth of imaginal tissues does not cease immediately upon heating, although they make but little progress. However, shortly after diapause has been re-established the new parts, of the brain at any rate, begin to degenerate. Perhaps they are already starting to readjust themselves to dormancy.

Discussion

More and more it is becoming possible to fit the widely assorted pieces of knowledge concerning diapause into an understandable picture. However, the incomplete picture resembles not so much a single unfinished jigsaw puzzle as the pieces from several puzzles jumbled together, many of them missing, others loose, and some of each assembled within the same frame.

Typically, it is at a rather early stage when an insect is fated either to enter diapause or to avoid it. Well before diapause is actually manifest the insect's development is switched onto the special line that leads to dormancy. If the work of Fukuda (12–15) on *Bombyx* proves generally applicable it will mean that the brain and endocrine organs control the switch-over as directed by the inheritance and environment of the insect. It is a period very sensitive to surrounding conditions.

Instead of development being directed primarily towards the maturation of imaginal organs, towards the rapid growth and differentiation of structures that will directly serve the purpose of adulthood and reproduction, development assumes another objective, the establishing of a protective dormant state. During dormancy an insect's body must be relatively self-sufficient. It must have within itself a large quantity of stored food and must be able to conserve its energy and water very efficiently. Consequently the stage is marked by resistance to desiccation and a low metabolic rate (associated with a breakdown of the cytochrome system). While the insect is preparing for diapause, imaginal maturation has to make way to some extent for the processes by which extra food reserves are built up and other preparations are made. A pronounced maturation deficit may result as it does in some Syrphidae (40, 41); in other cases the deficit is not so great. In *C. cinctus* comparable nondiapause forms were not available for comparison but since imaginal growth during diapause is not extensive the maturation deficit can hardly be great.

After the preparations are complete the insect finds itself in a state of relative inactivity in which it is prepared to weather the unfavorable season that should normally be drawing near. The insect has been committed to a period of development of a certain type, which may best be called diapause development (1). Diapause development is a slow process and characteristically has a lower optimum temperature than other phases of development. It must be completed before the kind of development that is usually regarded as ordinary can recommence. Its nature is not understood; either it may be a usual and necessary component of the development of all insect specimens that has become dissociated from the other components, or it may be a special process interjected only in diapause specimens.

During diapause the insect is "dormant" primarily because it lacks the GDH that is necessary to promote differentiation and stimulate the molting process, and diapause development seems essentially to serve the reactivation of the brain-prothoracic gland system. Had it not been for the intercession

of diapause this endocrine system would have been active long before and the more "usual" developmental processes would have continued uninterrupted.

The main question now is a double one. Why is the brain-prothoracic gland system inactive, and how does diapause development eventually terminate its inactivity? In attempting to find an answer it is important but most difficult to distinguish between factors directly relating to the inactivity and reactivation of the system and those that are only adjuncts to the dormant state. One of several possibilities is that diapause development constitutes a progressive change in the animal's general physiology that finally activates the brain. It is quite unlikely that the amount of body moisture is a critical factor (7). It is also unlikely that the growth of imaginal tissues is important; otherwise diapause insects with a maturation deficit would hardly develop normally when reactivated by an artificial supply of GDH. Where GDH is introduced early in diapause in any insect with an extreme maturation deficit, development may possibly not be normal. Generally, however, a maturation deficit and subsequent growth of anlagen during diapause are probably only a supplementary adaptation of the life cycle and are not related to the reactivation of the brain–prothoracic gland system. Andrewartha's (1) food mobilization hypothesis seems not unreasonable, but, again, food storage is accessory to diapause and its mobilization may well be incidental to the reactivation of the GDH system. Interaction between the two processes may not be without some influence, however.

Alternatively, diapause development may be a change within the brain and nervous system that makes them more receptive to potential stimuli. Towards the end of diapause the brain appears to be more receptive to stimulation, sometimes causing morphogenesis to occur prematurely. Again, diapause development may simply represent the manufacture and storage of neurohormone in the brain, to be released when the cells are full. The brain does secrete a neurohormone at the end of diapause that undoubtedly plays a part in reactivating the prothoracic glands, but it cannot be said definitely whether it is made during diapause development or at its end. Diapause development may, of course, be several such things combined. There is little to suggest which are important.

Possibly a clue to diapause development is to be found in the behavior of *C. cinctus* during the process of diapause reinstatement. Let it be assumed that appropriate heating stops most developmental processes, but does not prevent the neurohormone from being produced and then destroyed or dissipated. If the neurohormone is one of the main things responsible for the production of GDH and the resurgence of morphogenesis after diapause, and if it is lost before it can complete its task, then diapause must again intercede. A diapause reinstatement mechanism of this kind fits in best and most simply with a scheme of diapause development based on the production and gradual accumulation of neurohormone in the brain during the period of chilling. A reinstated diapause would continue until chilling permitted the brain to build

up another supply of neurohormone. So far, however, there is no evidence that there is actually an increase in the amount of stored neurohormone during the course of diapause development.

An alternative idea has already been proposed—that diapause development is some other physiological change in the insect and the brain responds by hormone production and secretion only at its completion; that the brain does not slowly produce neurohormone in direct response to chilling. If this is true, then diapause reinstatement must be a rather more complicated process. An effective heat exposure not only would have to let the neurohormone be destroyed but also must reverse the changes that had taken place during diapause. This hypothesis lacks the attractive simplicity of the other, but ultimately may prove the more realistic. The fact should be remembered that more than just a stimulus by the neurohormone is indicated in the activation of the prothoracic glands.

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References

1. Andrewartha, H. G. Diapause in relation to the ecology of insects. Biol. Revs. Cambridge Phil. Soc. 27 : 50-107. 1952.
2. Arvy, L. and Gabe, M. The intercerebralis-cardiacum-allatum system of some Plecoptera. Biol. Bull. 106 : 1-14. 1954.
3. le Berre, J.-R. Action de la température initiale d'incubation sur la diapause embryonnaire du criquet migrateur des landes. Compt. rend. 232 : 1870-1872. 1951.
4. Bodine, J. H. Hibernation and diapause in certain Orthoptera. III. Diapause—a theory of its mechanism. Physiol. Zoöl. 5 : 549-554. 1932.
5. Bounhiol, J.-J. L'achèvement de la métamorphose et la mue imaginale seraient commandés par le cerveau à la fin de la vie larvaire chez *Bombyx mori* L. Compt. rend. 235 : 671-672. 1952.
6. Bounhiol, J.-J. Nature probablement sécrétoire du facteur cérébral conditionnant la mue imaginale de *Bombyx mori* L. Compt. rend. 235 : 747-748. 1952.
7. Church, N. S. Moisture and diapause in the wheat stem sawfly, *Cephus cinctus* Nort. Can. Entomologist, 87 : 85-97. 1955.
8. Dawson, R. W. The problem of voltinism and dormancy in the polyphemus moth (*Telea polyphemus* Cramer). J. Exptl. Zool. 59 : 87-132. 1931.
9. Day, M. F. Possible sources of internal secretions in the heads of some holometabolous insects. Anat. Record, 78, Suppl. 264 : 150. 1940.
10. Dickson, R. C. Factors governing the induction of diapause in the oriental fruit moth. Ann. Entomol. Soc. Amer. 42 : 511-537. 1949.
11. Fukuda, S. The hormonal mechanism of larval molting and metamorphosis in the silkworm. J. Fac. Sci. Imp. Univ. Tokyo, Sect. IV, 6 : 477-532. 1944.
12. Fukuda, S. Function of the pupal brain and suboesophageal ganglion in the production of nondiapause and diapause eggs in the silkworm. Annot. zool. jap. 25 : 149-155. 1952.

13. FUKUDA, S. Determination of voltinism in the univoltine silkworm. Proc. Japan Acad. 29 : 381-384. 1953.

14. FUKUDA, S. Determination of voltinism in the multivoltine silkworm. Proc. Japan Acad. 29 : 385-388. 1953.

15. FUKUDA, S. Alteration of voltinism in the silkworm following transection of pupal oesophageal connectives. Proc. Japan Acad. 29 : 389-391. 1953.

16. GOMORI, G. Observations with differential stains on human islets of Langerhans. Am. J. Pathol. 17 : 395-406. 1941.

17. GRISON, P. Effets d'implantation de cerveaux chez le doriphore (*Leptinotarsa decemlineata* Say) en diapause. Compt. rend. 228 : 428-430. 1949.

18. HANSTROM, B. Neurosecretory pathways in the heads of crustaceans, insects and vertebrates. Nature, 171 : 72-73. 1953.

19. L'HÉLIAS, C. Étude des glandes endocrines postcérébrales et du cerveau de la larve des *Lophyrus pini* (L.) et *rufus* (André) (Diprionidae). Bull. soc. zool. France, 77 : 106-112. 1952.

20. L'HÉLIAS, C. Étude de la glande prothoracique chez la larve d'*Apis mellifica* (Hyménoptere). Bull. soc. zool. France, 77 : 191-195. 1952.

21. HOLMES, N. D. Morphology of the wheat stem sawfly, *Cephus cinctus* Nort. *In* Tech. Rept. of Field Crop Insect Sect., Science Service Lab., Lethbridge, Alta., for 1952. Unpublished.

22. KETCHEL, M. M. and WILLIAMS, C. M. The prothoracic gland hormone as a sustained stimulus for the growth and differentiation of insect tissues. Anat. Record, 117 : 542. 1953.

23. KOGURE, M. The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. J. Dept. Agr. Kyūshū Imp. Univ. 4 : 1-93. 1933.

24. KOPEC, S. Studies of necessity of the brain for the inception of insect metamorphosis. Biol. Bull. 42 : 322-432. 1922.

25. LEES, A. D. Entomology—the physiology of diapause. Science Progr. 38 : 735-742. 1950.

26. LEES, A. D. Entomology—diapause. Science Progr. 40 : 306-312. 1952.

27. LEES, A. D. The significance of the light and dark phases in the photoperiodic control of diapause in *Metatetranychus ulmi* Koch. Ann. Appl. Biol. 40 : 487-497. 1953.

28. MATTHÉE, J. J. The structure and physiology of the egg of *Locustana pardalina* (Walk.). Union S. Africa Dept. Agr. Sci. Bull. 316. 1951.

29. POSSOMPÈS, B. Rôle de cerveau au cours de la métamorphose de *Calliphora erythrocephala* Meig. Compt. rend. 231 : 594-596. 1950.

30. PREBBLE, M. L. The diapause and related phenomena in *Gilpinia polytoma* (Hartig). I. Factors influencing the inception of diapause. Can. J. Research, D, 19 : 295-322. 1941.

31. ROUBAUD, E. Étude sur le sommeil d'hiver préimaginal des muscides. Bull. biol. France et Belg. 56 : 455-544. 1922. *Reviewed in* Rev. Appl. Entomol. B, 11 : 55. 1923.

32. ROUBAUD, E. Asthénobiose et hibernation obligatoire provoquées chez *Phlebotomus papatasii* Scop. Bull. soc. pathol. exotique, 21 : 436-439. 1928.

33. SALT, R. W. Some effects of temperature on the production and elimination of diapause in the wheat stem sawfly, *Cephus cinctus* Nort. Can. J. Research, D, 25 : 66-86. 1947.

34. SALT, R. W. Field Crop Insect Sect., Science Service Lab., Lethbridge, Alberta. Unpublished data.

35. SCHARRER, B. The prothoracic glands of *Leucophaea maderae* (Orthoptera). Biol. Bull. 95 : 186-198. 1948.

36. SCHARRER, B. and SCHARRER, E. Neurosecretion. VI. A comparison between the intercerebralis-cardiacum-allatum system of the insects and the hypothalamo-hypophyseal system of the vertebrates. Biol. Bull. 87 : 242-251. 1944.

37. SCHARRER, E. The general significance of the neurosecretory cell. Scientia, 46 : 177-183. 1952.

38. SCHARRER, E. and SCHARRER, B. Neurosecretion. Physiol. Revs. 25 : 171-181. 1945.

39. SCHMIEDER, R. G. The control of metamorphosis in Hymenoptera. Anat. Record, 84 : 514. 1942.

40. SCHNEIDER, F. Beitrag zur Kenntnis der Generations-verhältnisse und Diapause räuberischer Schwebfliegen (Syrphidae). Mitt. schweiz. entomol. Ges. 21 : 249-285. 1948.

41. SCHNEIDER, F. Die Entwicklung des Syrphidenparasiten *Diplazon fissorius* Grav. Mitt. schweiz. entomol. Ges. 23 : 155-194. 1950.

42. SELLIER, R. Diapause larvaire et macroptérisme chez *Gryllus capestris*. Compt. rend. 228 : 2055-2056. 1949.

43. SELLIER, R. La glande prothoracique des gryllides. Arch. zool. exptl. et gén. Notes et Rev. 88 : 61-72. 1951.

44. SIMMONDS, F. J. The influence of maternal physiology on the incidence of diapause. Trans. Roy. Soc. (London), B, 233 : 385-414. 1948.

45. THOMSEN, E. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blowfly, *Calliphora erythrocephala* Meig. J. Exptl. Biol. 29 : 137-172. 1952.

46. VOGT, M. Induktion von Metamorphoseprozessen durch implantierte Ringdrüsen bei *Drosophila*. Wilhelm Roux' Arch. Entwicklungsmech. Organ. 142 : 131-182. 1942.

47. WIGGLESWORTH, V. B. The physiology of ecdysis in *Rhodnius prolixus*. II. Factors controlling moulting and "metamorphosis". Quart. J. Microscop. Sci. 77 : 191-222. 1934.

48. WIGGLESWORTH, V. B. The determination of characters at metamorphosis in *Rhodnius prolixus* (Hemiptera). J. Exptl. Biol. 17 : 201-222. 1940.

49. WIGGLESWORTH, V. B. The function of the corpus allatum in *Rhodnius prolixus*. J. Exptl. Biol. 25 : 1-14. 1948.

50. WIGGLESWORTH, V. B. Metamorphosis in insects. Proc. Roy. Entomol. Soc. London, C, 15 : 78-82. 1951.

51. WIGGLESWORTH, V. B. The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in molting. J. Exptl. Biol. 29 : 561-570. 1952.

52. WILLIAMS, C. M. Physiology of insect diapause. The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. Biol. Bull. 90 : 234-243. 1946.

53. WILLIAMS, C. M. Physiology of insect diapause. II. Interaction between pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. Biol. Bull. 93 : 89-98. 1947.

54. WILLIAMS, C. M. Physiology of insect diapause. III. The prothoracic glands in the cecropia silkworm, with special reference to their significance in embryonic and post-embryonic development. Biol. Bull. 94 : 60-65. 1948.

55. WILLIAMS, C. M. Extrinsic control of morphogenesis as illustrated in the metamorphosis of insects. Growth Symposium, 12 : 61-74. 1948.

56. WILLIAMS, C. M. Prothoracic glands of insects in retrospect and prospect. Biol. Bull. 97 : 111-114. 1949.

57. WILLIAMS, C. M. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. Biol. Bull. 103 : 120-138. 1952.

ESTIMATION OF EGG POPULATIONS OF THE LARCH SAWFLY, *PRISTIPHORA ERICHSONII* (HTG.)[1]

By W. G. H. Ives[2]

Abstract

The larch sawfly, *Pristiphora erichsonii* (Htg.), lays its eggs in the new terminal shoots of tamarack, *Larix laricina* (Du Roi) K. Koch. The oviposition injury usually causes the shoots to curl. During 1952 a sampling project was conducted in the Whiteshell Forest Reserve, Manitoba, to determine the feasibility of sampling tamarack trees to obtain estimates of the egg population of the larch sawfly. Additional data on the frequency distribution of the number of larch sawfly eggs per shoot were collected in 1953 and 1954 from several areas in Manitoba and Saskatchewan. The number of eggs per curled tip varied between plots and between trees on one plot, but no factors contributing to variation could be found. The frequency distribution of the number of eggs per curled shoot was found to be a modified logarithmic normal distribution. The number of curled tips per branch and the number of branches per crown level varied between crown levels and between tree types. Stratification of the sample increased the efficiency of sampling, reducing the standard error of the mean by about 15% and the required sample size by about 30%. The large variation in the estimated number of curled tips per tree indicates that a large sample of trees is required to obtain accurate estimates. As a compromise between accuracy and practicability it is recommended that six-branch samples be taken from each of at least 15 trees, using stratified sampling with proportional allocation. Simple random sampling, taking two branches from the mid-crown of at least 25 trees, is suggested to provide a population index of sufficient accuracy for survey purposes.

Introduction

Owing to its importance as a pest of tamarack (*Larix laricina* (Du Roi) K. Koch) the larch sawfly, *Pristiphora erichsonii* (Htg.), has received intensive investigation in recent years. Further detailed studies require more precise methods for estimating populations of various stages of the insect than are now available.

This paper introduces a method for sampling the egg stage. This stage is the simplest one on which to make population estimates because the eggs are inserted into the shoots of the current season's growth and egg scars may be counted after oviposition is complete. The oviposition scars usually cause the shoots to curl. The term curled tip will therefore be used in this paper to designate shoots bearing oviposition scars of the larch sawfly.

A later paper will consider the relationship between the number of eggs laid and the number of larvae completing development.

Methods

In 1952 two 0.6 acre plots were laid out in tamarack bogs in the Whiteshell Forest Reserve, Manitoba. A half-chain-interval grid was laid out in each plot. The trees were numbered with aluminum tags and their positions located on a map.

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Plot I was located in a mixed black spruce – tamarack stand, containing 175 tamarack trees with an average d.b.h. of 5.2 in. and an average height of 35 ft. Plot II was in a pure tamarack stand, containing 188 tamarack trees with an average d.b.h. of 5.1 in. and an average height of 38 ft. Both plots had an *Alnus rugosa* (Du Roi) Spreng. understory and a *Sphagnum* spp. ground cover.

The height of each tree was measured with an Abney level and the crown depth estimated. Each tree was also classified by crown shape, i.e. slender, medium, branchy, or stag-headed, and the number of branches in each crown level was estimated. Pole pruners marked off in 2-ft. intervals were used as a "measuring stick" and the number of branches in each third of the crown counted with the aid of binoculars. This estimate may be subject to error, but it was the only practical way to estimate the number of branches.

Six whole-branch samples were removed from each tree, two from each third of the crown. Some trees had so few branches that only two branch samples could be taken. In these instances the crown depths were not divided into thirds. Six-branch samples were taken from 156 trees in Plot I and 176 trees in Plot II. The crown depth of each tree was divided into thirds and random numbers drawn to determine the height and cardinal compass point from which each branch was to be sampled. The branch nearest a randomly selected location was sampled.

Extension pruners were used to remove the branches from the trees. The pruners consisted of 5-ft. sections, providing a moderately accurate estimate of the height of the cutting head, and the grid of twine furnished a check on the direction. The length of each branch removed, including an estimate of the length of stub left on the tree, and the number of shoots with and without oviposition scars were recorded.

Before the main sampling was initiated a random subsample of 10% of the trees on each plot was selected to provide an estimate of the mean number of eggs per curled tip. This subsample consisted of 17 trees in Plot I and 18 in Plot II. Two branches were taken from the three crown levels on each tree. It was originally planned to save five curled tips from each branch sampled, but many of the branches had less than five curled tips, so it was impossible to retain orthogonality in the data. A binocular microscope was used to count the number of egg scars on each shoot.

In 1953 all of the curled tips were collected from six trees in Whiteshell Forest Reserve, Manitoba, and examined under a binocular microscope to determine the number of egg scars per shoot.

Analyses and Discussion

Number of Eggs per Shoot

Analysis of Subsample

The mean number of eggs per curled shoot was estimated from the subsample data. The data were examined to determine if there was any variation in the number of eggs per shoot between branches, crown levels, or trees.

Complications introduced by the nonorthogonal nature of the data made it necessary to run a series of analyses to evaluate the influence of different factors on the number of eggs per shoot. The analysis for testing for differences between branches within crown levels was made from the pooled estimates of the variance between branches within crown levels and of the variance between tips within branches within crown levels. Crown level and tree classifications were disregarded. Twenty pairs of branches in Plot I and 30 pairs in Plot II were used for this analysis. The calculated F values were $F_{20,110} = .561$, and $F_{30,162} = .494$ respectively, which indicates no variation between branches; therefore the data were pooled before continuing the analyses.

To test for tree-by-crown-level interaction, the normal equations were solved for the estimates of the parameters. The sum of squares removed under the hypothesis of no interaction was then calculated for each plot, following the method of Kempthorne (3). The results are presented in Table I. The calculated F value for interaction was significant at the .01 level in Plot II, indicating interaction between trees and crown levels, but was not significant in Plot I.

To test for differences in the mean number of eggs per curled tip in each crown level an approximate test was used. For each tree the mean number

TABLE I

ANALYSIS OF VARIANCE FOR TESTING TREE-BY-CROWN-LEVEL INTERACTION IN NUMBER OF EGGS PER CURLED TIP

Source of variation	Degrees of freedom	Sums of squares	Mean square	
Plot I				
Fitting (μ, t_i, c_j)*	19	32115.954		
Difference	15	439.918	29.328	$F_{15,130} = 1.149$
Fitting (μ, t_i, c_j) and interaction	34	32555.872		
Within cells	130	3319.128	25.532	
Total	164	35875		
Plot II				
Fitting (μ, t_i, c_j)*	20	74353.953		
Difference	29	3056.040	105.381	$F_{29,193} = 2.224$**
Fitting (μ, t_i, c_j) and interaction	49	77419.946		
Within cells	193	9143.054	47.373	
Total	242	86563		

* μ = *over-all mean*; t_i = *tree effect* ($i = 1, 2, \ldots, n$); c_j = *crown level effect* ($j = 1, 2, 3$).

** *Throughout this paper a single asterisk after an F value indicates significance at the .05 level, and a double asterisk indicates significance at the .01 level.*

of eggs per curled tip for the upper crown level was paired with the middle crown level mean and then with the lower crown level mean. The t tests of the differences between means gave the results shown in Table II. The tests for differences between upper versus middle crown means and upper versus lower crown means are correlated. However, they do not utilize any assumption of zero interaction. None of these was significant, and the hypothesis that there were no differences between the three crown levels was accepted. Variation between trees was tested, using pooled data for each tree. This test procedure is insensitive and can introduce bias but the amount of bias was probably small. The between and within trees sums of squares were computed for each plot. The results of these analyses (Table III) show the calculated F value for Plot I to be significant at the .01 level, although the one for Plot II is not significant.

Three factors, crown shape (slender, medium, branchy, stag-headed), height (40 ft. and over, under 40 ft.), and crown depth (25 ft. and over, under 25 ft.), could influence the number of eggs per curled tip. The effect of each factor was tested for Plot I. The analyses were of the form: between classes, among trees within classes, between tips within trees within classes.

TABLE II

CALCULATED t VALUES FOR TESTING DIFFERENCES IN MEAN NUMBER OF EGGS PER SHOOT IN DIFFERENT CROWN LEVELS

	Plot I		Plot II	
Comparison	t	Degrees of freedom	t	Degrees of freedom
Upper vs. middle crown	1.29	7	0.21	14
Upper vs. lower crown	0.90	5	0.99	13

TABLE III

ANALYSIS OF VARIANCE FOR TESTING FOR DIFFERENCES BETWEEN TREES WITH RESPECT TO THE NUMBER OF EGGS PER CURLED TIP

Source of variation	Degrees of freedom	Sums of squares	Mean square	
Plot I				
Between trees	16	1475.765	92.235	$F_{16,\ 147} = 3.590^{**}$
Within trees	147	3776.790	25.692	
Total	163	5252.555		
Plot II				
Between trees	17	1037.514	61.030	$F_{17,224} = 1.116$
Within trees	224	12250.606	54.690	
Total	241	13288.120		

Satterthwaite's approximations for degrees of freedom and mean square were used to test for differences between classes. A circumflex is used to indicate the estimated values for degrees of freedom. The F values obtained were:

Between crown shape classes	$F_{3,16} = 2.153$
Between height classes	$F_{1,14} = 1.358$
Between crown depth classes	$F_{1,15} = 1.039$

None of these was significant at the .05 level. These tests are likely to be very insensitive. However, no basis for grouping the means could be found, so that the best estimate of the number of eggs per curled tip is the mean of the tree means for each plot. The standard error was computed from the sum of squares of the tree means (Table IV). These results indicate that a larger subsample might have been more informative, but the design of the sampling did not anticipate the large number of branches with less than five curled tips. A larger subsample might have revealed some further basis upon which to group the number of eggs per curled tip.

TABLE IV

MEAN NUMBER OF EGGS PER CURLED TIP

Plot	Mean	Standard error	95% confidence interval
I	14.334	.807	$12.752 < \mu < 15.916$
II	17.843	.518	$16.828 < \mu < 18.858$

Frequency Distribution

There is some evidence in the preceding section that the variation in number of eggs per shoot is greater within branches than between branches. Wallace (6) has shown that the length of shoots decreases after prolonged attack by the larch sawfly, and there is some indication that the number of eggs per shoot tend to be lower in areas that have been severely defoliated for a prolonged period. Examination of approximately 4500 tamarack shoots bearing oviposition scars of the larch sawfly provided information on the type of frequency distribution followed. The shoots were collected from six trees in the Whiteshell Forest Reserve, Manitoba, in 1953. All shoots bearing oviposition scars were collected from each tree.

The distribution of the number of eggs per shoot was plotted for each tree. All followed the same skewed distribution. The data were pooled and tested for normality by plotting the probits against the number of eggs (Line I, Fig. 1). The number of eggs per shoot is not normally distributed, since a relatively straight line is required to indicate normality.

The skewed distribution resembled that of a logarithmic normal. The agreement was tested by plotting the probits against the logarithms of the number of eggs per shoot. The adjustment given by Kenney and Keeping

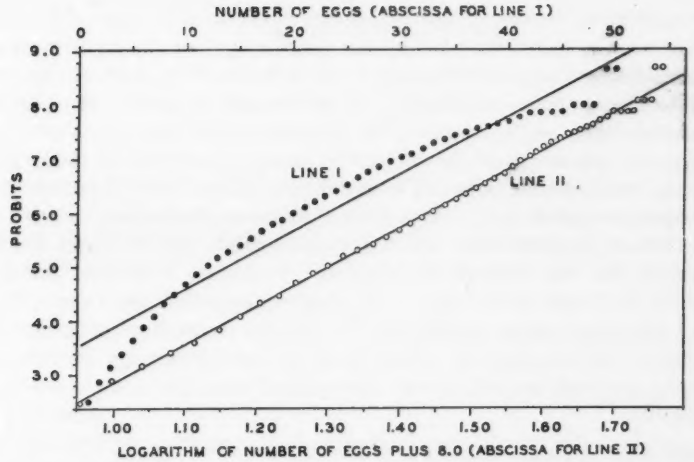

FIG. 1. Test for goodness-of-fit on number of eggs per curled shoot applied to data from six trees: Line I—test for normal distribution; Line II—test for logarithmic normal distribution.

(4) was necessary to straighten the line. The logarithms of the variable $y = x + i$ $(i = 1, 2, \ldots, n)$ were plotted against the probits, where x refers to the number of eggs per shoot. The variable $y = x + 8$ produced an approximately straight line (Line II, Fig. 1). The fit was tested with the Chi-square test for the logarithmic normal distribution (1). The value of χ^2 obtained was 35.978, with 38 degrees of freedom. Using the normal approximation for testing for significance, i.e., $t = \sqrt{2\chi^2} = \sqrt{2 \text{ d.f.} - 1}$, the probability of obtaining a larger value of χ^2 was found to be .5714, indicating a satisfactory fit to the observed data.

The number of egg scars per shoot was tested for data collected from four areas in 1953 and 1954, to determine if this transformation produced normality in the distribution. The data were collected by the Forest Insect Survey, Winnipeg, from a number of plots in the Northern and Prince Albert districts of Saskatchewan, and the Southeastern and Eastern districts of Manitoba. The numbers of shoots with egg scars examined from these areas were as shown in Table V.

TABLE V

NUMBER OF CURLED SHOOTS EXAMINED FOR EGG SCARS

Year	Area			
	Prince Albert, Saskatchewan	Northern Saskatchewan	Southeastern Manitoba	Eastern Manitoba
1953	471	210	335	278
1954	323	253	496	775

The agreement between the observed frequencies and the logarithmic normal distribution was tested by plotting the probits against the logarithms of the numbers of eggs plus eight for each area and year (Fig. 2). Numbers were added to the probits, as indicated in the figure, to permit the plotting of several tests on a single graph. The agreement with the logarithmic normal distribution appears good for all areas, when allowance is made for the relatively small sample sizes. These results indicate that the transformation produces approximate normality in the data over a wide area.

No data are available from which inferences can be drawn about the factors responsible for the skewed distribution. However, a skewed distribution would result if most of the eggs were laid when the shoots were short, if only a limited portion of most shoots was suitable for oviposition, or from a combination of the two factors. Additional factors relating to the behavior of the insect may influence the number of eggs laid per shoot, but no information is available.

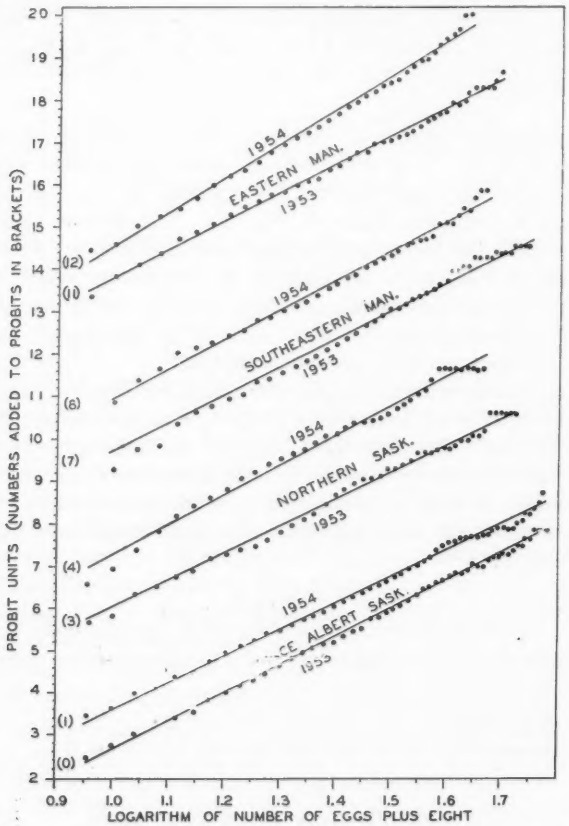

FIG. 2. Test for goodness-of-fit for logarithmic normal distribution on number of eggs per curled shoot applied to survey data.

Analysis of Main Sample

The only method of expressing the population density considered in the following analysis will be the number of curled tips or eggs per tree or unit area. Estimates of population density by this method are not influenced by the vigor of the trees but comparisons between estimates of expected defoliation in different stands are difficult to make because a given number of sawfly larvae would cause more defoliation on small trees than on large trees.

Bases for Stratification

Number of Curled Tips per Branch

The analysis of the data is mainly concerned with finding which factors, if any, affect the variability in the number of curled tips on a per branch and per tree basis. Variations in the number of curled tips per branch will be considered first.

Analyses of variance tests were made for each plot to determine whether there were any tree or crown level differences or tree-by-crown-level interaction (Table VI). In both cases the calculated F values were significant at the .01 level, indicating variation between trees and between crown levels and the presence of interaction. Although the F value for interaction was highly significant in both cases, it was not large, indicating that the interaction variance was rather small. For convenience the interaction was disregarded in the following analyses.

Although the two plots were fairly uniform, there was considerable variation between the trees on each plot. To try to obtain more homogeneous subsets of data the trees were classified according to crown shape, tree height, and

TABLE VI

ANALYSIS OF VARIANCE FOR TESTING FOR TREE EFFECTS, CROWN LEVEL EFFECTS, AND TREE-BY-CROWN-LEVEL INTERACTION BASED ON THE NUMBER OF CURLED TIPS PER BRANCH

Source of variation	Degrees of freedom	Sums of squares	Mean square	F
Plot I				
Between trees	155	21731.875	140.206	3.741**
Between crown levels	2	2130.506	1065.253	28.420**
Tree × crown level	310	15044.494	48.531	1.295**
Error	468	17541.500	37.482	
Total	935	56448.375		
Plot II				
Between trees	175	18566.143	106.092	2.634**
Between crown levels	2	1836.415	918.208	22.799**
Tree × crown level	350	19139.252	54.684	1.358**
Error	528	21264.500	40.274	
Total	1055	60806.310		

crown depth. All of these classifications are somewhat arbitrary. To test for interaction between the three criteria of classification used, the sums of squares removed under the hypothesis of no interaction and under the assumption of interaction were calculated for each plot. The difference between these sums of squares was compared to the error mean square by means of an F test. To calculate the sum of squares removed under the hypothesis of no interaction the normal equations were solved for estimates of the parameters involved. The interaction was not significant in Plot I but was significant at the .01 level in Plot II (Table VII) indicating interactions between the criteria used as the basis for stratification.

The effect of each of the factors used in stratifying the sample on the variation in the number of curled tips was tested, using the tree totals. Analyses for variation between and within crown shape classes, between and within height classes, and between and within crown depth classes were conducted. These tests did not utilize any assumption about interactions but were probably rather insensitive. The calculated F values are shown in Table VIII. These results indicate that the crown shape class and crown depth class provide a basis for stratification.

TABLE VII

ANALYSIS OF VARIANCE USED IN TESTING FOR THE PRESENCE OF INTERACTION BETWEEN STRATA, BASED ON SIX-BRANCH TREE TOTALS OF NUMBER OF CURLED TIPS

Source of variation	Degrees of freedom	Sums of squares	Mean square	F
Plot I				
Fitting (μ, t_i, h_j, c_k)*	6	131201.14		
Difference	5	3379.95	675.990	1.029
Fitting (μ, t_i, h_j, c_k) and interaction	11	134581.09		
Within strata	145	95269.91	657.034	
Total	156	229851		
Plot II				
Fitting (μ, t_i, h_j, c_k)*	6	218837.23		
Difference	6	9169.11	1528.185	3.262**
Fitting (μ, t_i, h_j, c_k) and interaction	12	228006.34		
Within strata	164	76840.66	468.541	
Total	176	304847		

* μ = *over-all mean*; t_i = *type effect* $(i = 1, 2, 3, 4)$; h_j = *height effect* $(j = 1, 2)$; c_k = *crown depth effect* $(k = 1, 2)$.

TABLE VIII

TESTS FOR FACTORS AFFECTING NUMBER OF CURLED TIPS PER SIX-BRANCH SAMPLE

Analysis	Plot I	Plot II
Between crown shape classes	$F_{3,152} = 14.564^{**}$	$F_{3,172} = 16.230^{**}$
Between height classes	$F_{1,154} = 0.330$	$F_{1,174} = 0.295$
Between crown depth classes	$F_{1,154} = 1.003$	$F_{1,174} = 9.902^{**}$

Number of Branches per Tree

Since the estimated total number of curled tips per tree also depends on the number of branches per tree their relation to possible stratification should be considered. The counts of the number of branches in each crown level may be subject to error since there was some difficulty in seeing the branches clearly through the foliage. However, since extreme care was exercised in the collection of the data, the error was considered negligible for the purposes of analysis.

An analysis of variance for testing variation in the number of branches on trees and at different crown levels was made for each plot. The tree-by-crown-level interaction was assumed negligible to provide an estimate of the variance. The calculated F values were all significant at the .01 level, indicating tree and crown level differences in both plots. A between and within class analysis was conducted for each of the criteria of tree classification in turn (Table IX). All the calculated F values are significant at the .01 level, confirming that the tree type has considerable influence on the number of branches.

The results of these analyses on number of curled tips and number of branches indicate that a reduction in variation between trees will be gained by stratification on the basis of tree crown shape class, height class, and crown depth class. The trees should also be sampled from each of the three crown levels.

TABLE IX

TESTS FOR FACTORS INFLUENCING NUMBER OF BRANCHES PER TREE

Analysis	Plot I	Plot II
Between crown shape classes	$F_{3,152} = 7.869^{**}$	$F_{3,172} = 17.125^{**}$
Between height classes	$F_{1,154} = 17.101^{**}$	$F_{1,174} = 31.123^{**}$
Between crown depth classes	$F_{1,154} = 47.616^{**}$	$F_{1,174} = 68.511^{**}$

Number of Curled Tips

Relationship to Number of Branches

The total number of curled tips in each crown level is estimated by multiplying the mean number of curled tips per branch by the number of branches. It is therefore important to know whether the mean number of curled tips per branch is independent of the number of branches. The correlation between

TABLE X

CALCULATED CORRELATIONS BETWEEN NUMBER OF BRANCHES AND MEAN NUMBER OF CURLED TIPS PER BRANCH

Class	Plot I				Plot II			
		r				r		
	d.f.	U.C.	M.C.	L.C.	d.f.	U.C.	M.C.	L.C.
S ⩾ 40 ⩾ 25	7	.421	.305	.454	27	.004	−.409*	.092
S ⩾ 40 < 25	—				4	−.378	.411	.834*
S < 40 ⩾ 25	15	.132	.235	−.514*	15	.076	.351	.229
S < 40 < 25	25	.136	.406*	.042	37	.371*	−.211	−.072
M ⩾ 40 ⩾ 25	29	−.045	−.117	−.196	28	.328	−.076	.158
M ⩾ 40 < 25	—				1	.756	−.573	.671
M < 40 ⩾ 25	13	.472	−.183	−.170	5	.799*	.231	−.191
M < 40 < 25	5	.395	−.001	.421	8	.308	.057	−.085
B ⩾ 40 ⩾ 25	7	−.307	−.366	−.337	15	.438*	−.113	.021
B ⩾ 40 < 25	—				—			
B < 40 ⩾ 25	5	−.477	−.479	−.442	3	.105	.366	−.782
B < 40 < 25	—				2	.315	−.365	−.751
St < 40 ⩾ 25	4	.129	−.373	−.216	—			
St < 40 < 25	24	.077	.318	−.316	7	.770*	−.163	.251
Average r		.100	−.024	−.049		.300	−.111	.060

NOTE: *U.C.—upper crown level; M.C.—middle crown level; L.C.—lower crown level. Crown shape: S—slender, M—medium, B—branchy, St—stag-headed.*

the mean number of curled tips per branch and the number of branches in each crown level was calculated for each stratum (Table X), assuming that the branch counts represent the true values.

Some of the calculated r values are significant at the .05 level, but none are significant at the .01 level. Fisher's z transformation, i.e. $z = \frac{1}{2}[\log_e(1 + r) - \log_e(1 - r)]$, was used to test whether the r values could have arisen from a population with a common correlation. The test was applied to each crown level in each plot. A chart was used to make the transformation (5). None of the calculated χ^2 values were significant, indicating that the sample correlations in each crown level could have arisen from a population with a common correlation. The largest average r value is .300. This indicates that the number of branches and the mean number of curled tips per branch are essentially uncorrelated in each crown level.

Differences Between Crown Levels

The correlation between the true means for the number of curled tips per branch in each of the three crown levels may be estimated by utilizing the formula

$$\text{correlation} = \frac{\text{covariance}}{\sqrt{\text{product of variances}}}.$$

The covariance between the observed crown level means is an unbiased estimate of the covariance between the true means. The estimates of the variances of

TABLE XI

ESTIMATES OF VARIANCES OF THE TRUE MEANS, COVARIANCES OF THE OBSERVED MEANS, AND ESTIMATES OF THE CORRELATIONS BETWEEN THE TRUE MEANS FOR NUMBER OF CURLED TIPS PER BRANCH IN DIFFERENT CROWN LEVELS

	Estimate*								
				Covariances					
Plot	$\hat{\sigma}_1^2$	$\hat{\sigma}_2^2$	$\hat{\sigma}_3^2$	$\bar{x}_1, \bar{x}_2$	$\bar{x}_1, \bar{x}_3$	$\bar{x}_2, \bar{x}_3$	$\hat{\rho}_{12}$	$\hat{\rho}_{13}$	$\hat{\rho}_{23}$
I	5.60	15.24	41.57	10.62	13.01	22.22	1.15	0.85	0.88
II	6.53	10.53	30.26	5.92	5.57	14.22	0.71	0.40	0.80

** The subscripts 1, 2, and 3 refer to the upper, middle, and lower crown levels respectively.*

the true means may be obtained by equating observed and expected mean squares in the components of variance models (5). The analyses were of the form: between trees, and between branches within trees. An analysis was made for each crown level in each plot. The estimates of the variances of the true means, of the covariance of the observed means, and of the correlations between the true means are shown in Table XI. These are consistent estimates of the correlation, but they are subject to an unknown amount of bias, which is exemplified by the estimated correlation that is greater than unity.

Estimated Number of Curled Tips per Tree

The estimate of the mean number of curled tips per tree that will be considered is:

$$T = \frac{1}{n} \sum_{i=1}^{n} \sum_{j=1}^{3} y_{ij}\, x_{ij.2}$$

where n is the number of trees in the sample,

y_{ij} is the number of branches in the j^{th} crown level of the i^{th} tree (assumed to be without error),

and $x_{ij.} = \frac{1}{2} \sum_{k=1}^{2} x_{ijk}$ is the observed mean number of curled tips per branch in the j^{th} crown level of the i^{th} tree.

The estimated number of curled tips and number of eggs per tree in the different classes are shown in Table XII.

The variance and estimated variance for the above expression were then derived, ignoring the finite population correction. This correction will be small for the intensity of sampling which is feasible. The variance of the mean number of curled tips per tree will be estimated by:

$$\hat{V}(T) = \frac{1}{n}\left[\hat{\sigma}_t^2 + \frac{1}{k} \sum_{j=1}^{3} \left(\frac{1}{n} \sum_{i=1}^{n} y_{ij}^2\right) s_j^2\right],$$

TABLE XII

ESTIMATED MEAN NUMBER OF CURLED TIPS AND EGGS IN EACH CLASS OF TREES (n_i REFERS TO THE NUMBER OF TREES)

Class	Plot I			Plot II		
	n_i	Curled tips	Eggs	n_i	Curled tips	Eggs
S ≥ 40 ≥ 25	9	236	3384	29	383	6839
S ≥ 40 < 25	0	—	—	6	108	1925
S < 40 ≥ 25	17	164	2354	17	377	6720
S < 40 < 25	27	110	1571	39	263	4691
M ≥ 40 ≥ 25	31	341	4888	30	590	10524
M ≥ 40 < 25	0	—	—	3	231	4129
M < 40 ≥ 25	15	278	3982	7	455	8127
M < 40 < 25	7	305	4375	10	500	8920
B ≥ 40 ≥ 25	9	668	9578	17	791	14107
B ≥ 40 < 25	0	—	—	0	—	—
B < 40 ≥ 25	7	684	9804	5	1485	26500
B < 40 < 25	2	653	9364	4	466	8322
St < 40 ≥ 25	6	962	13795	0	—	—
St < 40 < 25	26	304	4359	9	269	4803
Over-all mean		326	4670		457	8149

where n is the number of trees in the sample,
k is the number of branches sampled per crown level,
s_j^2 is the between branches within trees mean square in the analysis of variance table for the j^{th} crown level,
$\sum_{i=1}^{n} y_{ij}^2$ is the sum of the squares of the number of branches in the j^{th} crown level of the trees sampled,
and $\hat{\sigma}_t^2$ = mean square between estimated tree totals

$$= \sum_{j=1}^{3} \left(\frac{1}{kn} \sum_{i=1}^{n} y_{ij}^2 \, s_j^2 \right) .$$

The values of these quantities are shown in Table XIII. The estimated variances for the estimated number of curled tips per tree, on Plots I and II, are 824.537 and 865.264 respectively. In areas similar to those dealt with here, the respective parts of the formulas for the two plots may be averaged to give the variance with n trees, and k branches sampled per crown level, as:

$$\hat{V}(T) \backsimeq \frac{1}{n} \left(100164 + \frac{80789}{k} \right) .$$

Random Versus Stratified Sampling

Although stratification seems justified, the allocation of sampling units may be difficult if stratified sampling with proportional allocation is used, especially if the sample size is small. It would therefore be instructive to compare the size of the confidence intervals for the mean number of curled tips per tree in each plot, and sample sizes needed for a desired degree of accuracy, for each type of sampling.

TABLE XIII

QUANTITIES USED IN CALCULATING THE VARIANCE OF THE MEAN NUMBER OF CURLED TIPS PER TREE

	Plot I		Plot II	
	s_i^2	$\frac{1}{n}\sum_{i=1}^{n} y_{ij}^2$	s_i^2	$\frac{1}{n}\sum_{i=1}^{n} y_{ij}^2$
Upper crown	11.160	1723.1	14.923	1702.4
Middle crown	27.016	898.6	43.358	835.7
Lower crown	74.269	421.4	62.540	395.4
Observed mean square (Est. tree totals)	128628		152286	
n	156		176	
$\hat{\sigma}_i^2$	91227		109101	

The confidence interval and required sample size may be estimated if the mean number of curled tips per tree is assumed to be approximately normally distributed. The variance of the sample mean for stratified sampling with proportional allocation with k strata is (neglecting finite corrections):

$$\text{variance} = \sum_{i=1}^{k} \frac{n_i}{n^2} \sigma_i^2 .$$

An unbiased estimate of this is $\sum_{i=1}^{k} \frac{n_i}{n^2} s_i^2$. The structure of the analysis of variance table for between and within strata is:

Source of variation	Degrees of freedom	Sums of squares	Mean square	Estimated mean square
Between strata	$k-1$	$\sum_{i=1}^{k} n_i (x_{i.} - x_{..})^2$	B	
Within strata	$\sum_{i=1}^{k} (n_i - 1)$	$\sum_{i=1}^{k}\sum_{j=1}^{n_i} (x_{ij} - x_{i.})^2$	W	σw^2
Total	$n-1$	$\sum_{i=1}^{k}\sum_{j=1}^{n_i} (x_{ij} - x_{..})^2$		

where x_{ij} is the estimated tree total for the j^{th} tree in the i^{th} strata. If σ_i^2 is not constant,

$$W = \sum_{i=1}^{k}\sum_{j=1}^{n_i} \frac{(x_{ij} - x_{i.})^2}{n-k} = \sum_{i=1}^{k} \frac{(n_i - 1)}{n-k} s_i^2 \cong \sum_{i=1}^{k} \frac{n_i}{n} s_i^2 ,$$

provided n_i is large and k is not too large (2). Therefore an approximate estimate of the variance of the mean number of curled tips per tree, using

TABLE XIV

STANDARD ERRORS AND CONFIDENCE INTERVALS FOR MEAN NUMBER OF CURLED TIPS PER TREE USING SIMPLE RANDOM SAMPLING AND STRATIFIED SAMPLING WITH PROPORTIONAL ALLOCATION

Type of sampling	Plot I		Plot II	
	$s_{\bar{x}}$	95% C.I.	$s_{\bar{x}}$	95% C.I.
Random	28.715	$268 < \mu < 383$	29.415	$398 < \mu < 516$
Stratified	25.072	$276 < \mu < 376$	23.587	$410 < \mu < 504$

TABLE XV

APPROXIMATE SAMPLE SIZES NEEDED TO ESTIMATE MEAN NUMBER OF CURLED TIPS PER TREE WITH VARIOUS DEGREES OF ACCURACY, USING SIMPLE RANDOM SAMPLING AND STRATIFIED SAMPLING WITH PROPORTIONAL ALLOCATION

Confidence level	Plot I			Plot II		
	$\lvert d \rvert$*	Random	Stratified	$\lvert d \rvert$*	Random	Stratified
.95	33	473	360	46	288	185
.90	66	80	61	92	50	32
.80	82	32	24	114	20	13
.80	108	18	14	152	11	7

* *The size of the half confidence interval width based on 10, 20, 25, and 30% of the observed mean for Plots I and II respectively.*

proportional allocation in stratified sampling, is provided by W/n. This approximation was used in calculating the variance for the stratified sampling. The standard error of the mean and the 95% confidence intervals for the mean number of curled tips per tree in each plot, using simple random sampling and stratified sampling with proportional allocation, are given in Table XIV. The approximate sample sizes needed for various degrees of accuracy, using the two types of sampling described above, are shown in Table XV. These sample sizes assume the same sampling method as used in this experiment, and are based on the observed tree total means, making use of the relation $d < s_{\bar{x}} t_\alpha$, which gives $n_0 = s^2 t_\alpha^2 / d^2$, where d is the half confidence interval width, $s_{\bar{x}} = s/\sqrt{n}$ is the standard error of the mean, and t_α is Student's t for the α confidence level, based on infinite degrees of freedom.

These results indicate that an increase in efficiency is obtained by the use of stratified sampling. Stratified sampling reduced the standard error of the mean by about 13% in Plot I and by about 20% in Plot II. Required sample sizes were reduced by about 25% for Plot I and 35% for Plot II by using the stratified sampling method. The greater efficiency must be considered in relation to the extra work involved. The time required to determine the

proportion of the various classes of trees in a stand might well outweigh the greater accuracy of the stratified sampling. However, if accurate estimates are required it is recommended that stratified sampling be used. The number of trees that can be sampled will be limited by time and labor. A sample of 15 or 20 trees per plot, using stratified sampling, will probably give reasonably accurate estimates with a moderate amount of effort.

Four-branch Samples per Crown Level

The above results on estimated sample sizes indicate that large samples are required for a reasonable degree of accuracy, using the present sampling method. The possible reduction in variance that could be obtained by sampling four branches per crown level may be estimated by utilizing the expression for the variance of the estimated tree totals. The variance for a sampling method, using four branches per crown level, may be calculated by assuming that the between sample and within sample mean squares will remain approximately the same. The estimated variances of the mean number of curled tips per tree, sampling two and four branches per crown level, and the per cent reduction obtained by sampling four branches per crown level are as follows:

	Plot I	Plot II
Two-branch samples	824.537	865.264
Four-branch samples	704.664	742.578
% reduction in variance	14.5	14.2

Since the work involved in taking four-branch samples from each crown level, for a given number of trees, is almost twice that required for two-branch samples, the reduction in variance is relatively small. Assuming that the mean squares remain reasonably constant, a reduction in the variance of the mean number of curled tips per tree of about 50% would be expected if two branches were sampled from each crown level of twice as many trees. Therefore it appears that it would not be feasible to sample more than two branches per crown level in an attempt to reduce the variance. In fact, it might be advisable to sample only one, although no estimate of the within crown level variance would then be available.

Sampling from the Mid-crown Only

Sampling from the upper third of the crown is somewhat impractical for survey purposes because of mechanical difficulties. This suggests the possibility that sampling of the lower or middle crown might give essentially the same estimate as obtained by sampling the complete crown. Field observations indicate that the lower crown is too variable to be satisfactory. The mid-crown therefore seems to be the most satisfactory portion to sample if only one crown level is to be used. The estimate of the total based on

mid-crown sampling only was obtained similarly to that obtained for whole trees, except that the mean number of curled tips per branch for the mid-crown was multiplied by the total number of branches on a tree. The difference between these two estimates was used as a sample from a population of differences and tested by means of Student's t test for paired differences (Table XVI). The mean difference is significant at the .05 level for Plot II, but not in the other two cases. These results indicate that mid-crown sampling will probably tend to overestimate the number of curled tips per tree. Whole tree sampling is therefore recommended when an absolute population estimate per tree is required.

For surveys the primary requisite is a population index, and for this purpose mid-crown sampling should be satisfactory. The mean number of curled tips or eggs per two-branch sample would be the simplest index to use since no branch counts need be made. Suitable randomization techniques would be required, to avoid personal bias in the selection of the branches. The approximate sample size needed for a given degree of accuracy may be

TABLE XVI

COMPARISON OF ESTIMATED NUMBER OF CURLED TIPS PER TREE, BASED ON WHOLE TREE SAMPLING AND MID-CROWN SAMPLING

	1952		Additional data collected in 1953
	Plot I	Plot II	
Mean difference*	36.36	93.67	266.50
Degrees of freedom	148	175	5
$s_{\bar{x}}$	23.17	26.62	119.46
Student's t	1.57	3.52	2.23
95% C.I.**	$-9.42 < \mu < 82.14$	$42.12 < \mu < 146.22$	$-40.63 < \mu < 573.63$

* *Mid-crown minus whole tree.*
** *.95 confidence interval on mean difference.*

TABLE XVII

APPROXIMATE SAMPLE SIZES NEEDED TO ESTIMATE THE MEAN NUMBER OF CURLED TIPS PER BRANCH, SAMPLING TWO BRANCHES FROM THE MID-CROWN OF EACH TREE

Confidence level	Plot I		Plot II	
	$\lvert d \rvert$*	Sample size	$\lvert d \rvert$*	Sample size
.95	0.80	690	1.24	322
.90	1.60	122	2.48	57
.80	2.00	47	3.09	22
.80	2.40	33	3.71	15

* *The size of the half confidence interval width based on 10, 20, 25, and 30% of the observed means for Plots I and II respectively.*

estimated, if the mean number of curled tips per two-branch sample from the mid-crown is assumed to be approximately normally distributed. The estimated number of trees needed to be sampled in Plots I and II, using simple random sampling, were calculated (Table XVII). These results indicate that large sample sizes are again required if a reasonable degree of accuracy is required. Stratification would probably reduce the required sample sizes appreciably, but would not be practical for survey purposes. A simple random sample of two branches from the mid-crown of 25 or 30 trees per plot is suggested to provide a population index. This index will possess only moderate accuracy, but a larger sample would not be practical.

Curled Versus Total Tips

The foregoing analyses have been concerned with estimates of the total numbers of curled tips on the branches or trees, and have not considered the ratio of curled tips to total tips. If the percentage of curled tips to total tips is to be used as a population index, it is important to know whether there are any crown level trends in the distribution of curled tips in relation to total tips. Because of the high variation encountered between individual branches, the data were grouped into nine classes according to crown shape and crown level for Plot I and Plot II and for additional data collected in 1953. These classes were slender, medium, and branchy trees and upper, middle, and lower crown levels. Both curled tips and total tips appeared to follow the same type of distribution. To test the hypothesis that the number of curled tips depends upon the total shoots available for oviposition, and not upon their height from the ground, the correlation between the number of curled tips and total shoots was calculated for each of the crown levels, using the grouping previously mentioned. The correlations obtained were .861, .756, and .747 for the upper, middle, and lower third of the crown respectively. Each of these correlations had seven degrees of freedom. The first is significant at the .01 level, and the others at the .05 level. The z transformation was used to determine if the correlations could have arisen from a population with a common correlation (5). The value of χ^2 obtained was .411, with two degrees of freedom. The probability of obtaining a larger value of χ^2 is approximately .80, so that there are no grounds for rejecting the hypothesis of common correlation. The average z is 1.081, which gives an average r of .794. From this it is concluded that the number of curled tips is related to the number of shoots available for oviposition and is not materially influenced by their height above the ground.

Modifications in Procedure

The method used for counting branches could be modified to reduce error. Counts made in the spring, when the buds had just broken, would be more accurate. The new foliage would identify the living branches but would not obstruct vision for counting branches on the far side of the crown. In an intensive study, a portable platform would greatly increase accuracy in counting branches.

The number of eggs per tree might be estimated by counting the number of eggs per branch sample, instead of the number of curled tips. However, the data for calculating confidence intervals and estimated sample sizes were not collected for this estimate.

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References

1. CROXTON, F. E. and COWDEN, D. J. Applied general statistics. Prentice-Hall, Inc., New York. 1947.
2. JESSEN, R. J. Unpublished lecture notes. Iowa State College, Ames, Iowa. 1951.
3. KEMPTHORNE, O. The design and analysis of experiments. John Wiley & Sons, Inc., New York. 1952.
4. KENNEY, J. F. and KEEPING, E. S. Mathematics of statistics. Pt. II, 2nd ed. D. Van Nostrand Company, Inc., New York. 1951.
5. SNEDECOR, G. W. Statistical methods. 4th ed. Iowa State College Press, Ames, Iowa. 1946.
6. WALLACE, D. R. Some reactions of larch trees to larch sawfly defoliation. Can. Dept. Agr. Forest Biol. Div. Bi-monthly Prog. Rept. 10, (1) : 2. 1954.

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Contents